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Crop pest control and pollination,
volume II, 2nd Edition

Edited by
Fang Ouyang, Xingyuan Men, Wen Xie and Wei Li

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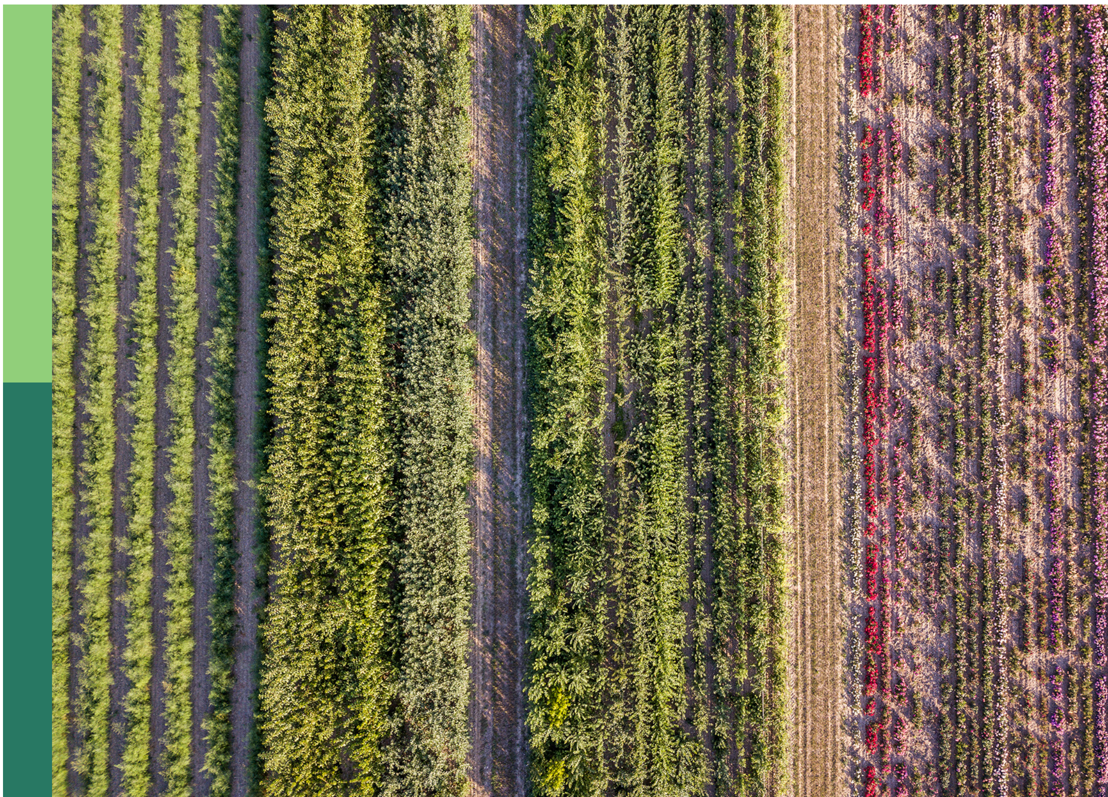

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Crop pest control and pollination volume II, 2nd Edition

Topic editors
Fang Ouyang — Institute of Zoology, Chinese Academy of Sciences (CAS), China
Xingyuan Men — Institute of plant protection, Shandong Academy of Agricultural Sciences, China
Wen Xie — Insititute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, China
Wei Li — Hunan Agricultural University, China

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Editorial: Crop pest control and pollination, volume II

Xingyuan Men[1]*, Wen Xie[2]*, Fang Ouyang[3] and Wei Li[4]

[1]Institute of Plant Protection, Shandong Academy of Agricultural Sciences, Jinan, China, [2]Institute of Vegetables and Flowers, Chinese Academy of Agricultural Science, Beijing, China, [3]State Key Laboratory of Integrated Management of Pest and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [4]College of Plant Protection, Hunan Agricultural University, Changsha, China

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EDITED AND REVIEWED BY
David Gonthier,
University of Kentucky, United States

*CORRESPONDENCE
Xingyuan Men
✉ menxy2000@hotmail.com
Wen Xie
✉ xiewen@caas.cn

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Editorial on the Research Topic
Crop pest control and pollination, volume II

In the fascinating realm of agricultural and ecological sciences, a diverse array of organisms play critical roles in maintaining the balance of ecosystems and influencing agricultural productivity. Crops, microbes, bioactive volatiles, nematodes, natural enemy insects, and native pollinators are all key players that impact pests and diseases, biological control, and pollination. Each of these organisms contributes to the intricate web of interactions that shape our environment and influence food production. Exploring the intricate relationships between microbial agents, bioactive volatiles, nematodes, insect-mediated biological control, and pollinators offers a wealth of potential in pest and disease management, promoting sustainable agriculture, and maintaining ecosystem health. In this Research Topic we explore research that harnesses these natural interactions to create more resilient and environmentally friendly approaches to meeting our agricultural and ecological challenges.

Microbes such as bacteria and fungi hold immense potential in controlling pests and diseases (da Silva Folli-Pereira et al., 2022). These strains produce natural substances that can act as deterrents, toxins, or even attractants for harmful insects or pathogens. Harnessing these microbial properties offers a sustainable and environmentally friendly alternative to traditional chemical pesticides, reducing the dependence on synthetic compounds and minimizing negative impacts on ecosystems. *Rhodopseudomonas palustris* belongs to Photosynthetic bacteria, which can decompose various carbon and nitrogen sources, promote plant growth in the rhizosphere of plants, and antagonize crop pathogens. Luo et al. reported that the *R. palustris* strain PSB06 increased the pepper yield by 33.45%, soil nitrogen concentration, and improved pepper rhizosphere bacterial α diversity. Wu et al. reported that the control efficiency of *R. palustris* strain PSB-06 combined with reduced amount of isoprothiolane, a fungicide against rice blast, was higher than the individual efficiency of fungicide, which highlighted the synergistic potential of PSB-06 to control rice blast, providing environmental protection and reducing the use of fungicides.

Bioactive volatiles, volatile organic compounds emitted by plants and microbes, also play a significant role in mediating interactions between organisms. These compounds can serve as chemical cues, attracting or repelling specific insects or other organisms (Das et al., 2013). Bioactive volatiles offer a complex communication network that can be harnessed to manipulate the behavior and distribution of pests or beneficial insects. The South American tomato pinworm, *Tuta absoluta,* is one of the most devastating invasive pests of solanaceous plants worldwide. *T. absoluta* is resistant to many chemical classes of insecticides, leaving producers with few chemical control options. Chen T. et al. reported that they found two attractants and two repellents for *T. absoluta* from plant released volatiles, which could be useful to develop the trapping and monitoring technology. Guo et al. reported that the microbe *Penicillium digitatum* was an important driver of the interactions between the yellow peach moth *Conogethes punctiferalis* and apple by altering apple volatiles, and these findings may form the basis for developing attractant baits for field trapping the yellow peach moth in the future.

Nematodes, small roundworms that inhabit soil ecosystems, represent another growing biological control option given that some are parasitic to insect pests. These beneficial nematodes can be used as biocontrol agents to control a variety of soil-dwelling insects due to their superior ability to actively search for hosts. By employing nematodes, we can enhance pest control measures while reducing the need for potentially chemical interventions. Root-feeding white grubs are one of the most serious pests of honeysuckle trees (*Lonicera japonica*) in China, Li et al. reported that entomopathogenic nematodes could provide curative efficacy against white grubs and significantly reduce honeysuckle plant death, which provides a biological control method for underground pests of Chinese medicinal materials.

Insect-mediated biological control is a strategy that leverages the natural relationships between insects to combat pests. Encouraging the presence and abundance of these natural enemies plays a crucial role in reducing pest populations and managing crop damage without relying on chemical interventions. Understanding the factors that affect insect predator populations, including temperature stress and habitats, is crucial for developing conservation strategies and managing biological control services effectively. Ren et al. reported that temperature not only influenced the population growth parameters, but also the predation rates of *Orius strigicollis* to the western flower thrips *Frankliniella occidentalis*. These results indicated that temperature should be taken into account when releasing *O. Strigicolli* to control *F. Occidentalis*. It is known that diversifying crop species within fields can create a more balanced ecosystem and enhance the biological control of natural enemies to reduce the pest outbreaks. Cui et al. reported that the habitat of natural enemies can be expanded for their preferences to feed and oviposit on different plants to achieve pest control in adjacent cropping systems.

Crop plants serve as both the host, non-host and habitat of various insect pests. Improving crop resistance and modifying the composition of the agricultural landscape are both effective strategies to reduce the harm caused by pests. Chen L. et al. reported that amending soils with a 30:1 ratio of bamboo charcoal and coconut bran induced biosynthesis of flavonoids, terpenoids, and phenolic acids in tomato plants, which improved plant growth and tolerance against South American tomato pinworm, thus reducing the survival of this destructive pests. Modifying the landscape around crop fields can help disrupt the breeding and survival of insect pests. Wen et al. reported that compositional heterogeneity positively affected an invasive pest *Bactrocera dorsalis* and its associator *Drosophila melanogaster*, whereas configurational heterogeneity negative affected *B. dorsalis*. The relative effects of landscape structures are consistent across multiple scales. These results provide new insights into landscape effects on interconnected species using a diverse spatial-scale approach.

Pollinators, such as bees, butterflies, and hoverflies, are vital for successful plant reproduction and food production for 87% of the leading global food crop species (Klein et al., 2007). Understanding the factors that affect pollinator populations, including temperature stress, is crucial for developing conservation strategies and managing pollination services effectively. Native pollinators play a crucial role in pollination, especially in ecosystems where they have co-evolved with native plant species. The decline of native pollinators due to habitat loss, pesticide use, and climate change poses significant challenges to agriculture and biodiversity. Recognizing and promoting the importance of pollinators is crucial to ensure the pollination and reproduction of native plants, maintain biodiversity, and sustain food production. Cortés-Rivas et al. reported that some native bee species can greatly improve the fruit set and fruit quality of the highbush blueberry cultivars in Chile. This research indicates that conservation of native pollinators, would improve blueberry fruit quality and is likely to improve overall crop productivity. The population of *Osmia excavata*, an important native pollinator in China, has been in serious decline over recent years. Song et al. reported that *O. excavata* had a low tolerance to high-temperature stress, and the larvae of *O. excavata* were more sensitive to temperature stress than adults, which provided evidence of causes that could be contributing to the population decline of *O. excavata*. Hoverflies have unique lifecycles that include a larval stage during which they consume aphids and other small insects, benefiting crop health. Cao et al. reported the morphological characteristics and life cycle of the hoverfly *Eristalinus arvorum*, a pollinating agent for crops and flowering plants widely distributed across Chinese agricultural and natural ecosystems. Monitoring and sampling of pollinators allow researchers and practitioners to assess the diversity, abundance, and health of these important species in agricultural landscapes. In oilseed rape fields in China, Shi et al. reported flight interception traps had a greater sampling efficiency than pan traps shedding light on strategies for efficiently monitoring agroecosystems.

Based on these findings in this Research Topic, harnessing the interactions between crops, microbes, beneficial nematodes, natural insect enemies and pollinators in agricultural systems, holds immense potential for agricultural ecosystem service, such as crop production, pest control and crop pollination. Encouraging the presence and manipulating abundance of these beneficial organisms, such as beneficial nematodes, microbes, natural enemies plays a vital role in reducing pest populations and crop damage without relying on chemical interventions. Additionally, methods like enhancing crop resistance and modifying habitats offer effective means to control insect pests. The utilization of bioactive volatiles provides a sophisticated communication network for manipulating the behavior and distribution of pests and beneficial insects. Furthermore, understanding the factors impacting natural enemy and pollinator populations, is essential for developing effective conservation and utilization strategies. By harnessing these natural interactions, sustainable agricultural systems can be created, promoting ecological balance and reducing reliance on chemical interventions.

Author contributions

XM: Funding acquisition, Writing – original draft, Writing – review & editing. WX: Writing – original draft, Writing – review & editing. FO: Writing – review & editing. WL: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Combining the microbial agent *Rhodopseudomonas palustris* strain PSB-06 with fungicides for controlling rice blast

Xiyang Wu[1,2], Yue Chen[1,2]*, Chunyan Chen[2], Qiang Huang[2], Yingfei Qin[1,2], Xin Zhang[2], Chenggang Li[2], Xinqiu Tan[1,2], Yong Liu[1,2]* and Deyong Zhang[1,2]*

[1]LongPing Branch, College of Biology, Hunan University, Changsha, China, [2]Institute of Plant Protection, Hunan Academy of Agricultural Sciences, Changsha, China

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EDITED BY
Wei Li,
Hunan Agricultural University, China

REVIEWED BY
Delong Li,
Qingdao Agricultural University, China
Weixiao Yin,
Huazhong Agricultural University, China
Yu Chen,
Anhui Agricultural University, China

*CORRESPONDENCE
Yue Chen
hnsfcy@126.com
Yong Liu
haoasliu@163.com
Deyong Zhang
dyzhang78@163.com

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The rice blast disease caused by *Magnaporthe oryzae* threatens global rice production yields. Tricyclazole and isoprothiolane are widely used fungicides with high activity against rice blast, and our previous study indicated the photosynthetic bacterium *Rhodopseudomonas palustris* PSB-06 significantly antagonizes rice blast. However the effect of combining these two chemical fungicides with PSB-06 on rice blast control is unclear. Here we test the control effect of photosynthetic bacteria PSB-06 combined with isoprothiolane and tricyclazole on rice blast. The growth of PSB-06 was unaffected by up to 1.25 mg/L of tricyclazole and 0.3 mg/L of isoprothiolane in the photosynthetic medium, indicated the two fungicides have no inhibition on PSB-06. The control efficiency in the field test reached 76.06% when PSB-06 was combined with isoprothiolane. This value was significantly higher than the individual efficiency of PSB-06 (67.99%) and tricyclazole (65.46%) and the combined control efficiency (72.20%) of those two antifungal agents. Our current findings highlighted the potential of combining *R. palustris* strain PSB-06 with isoprothiolane to control rice blast, providing environmental protection and reducing the use of fungicides.

KEYWORDS

***Magnaporthe oryzae*, *Rhodopseudomonas palustris*, tricyclazole, isoprothiolane, combined application**

Introduction

Rice blast is an important fungal disease causing significant annual losses to global rice production. It is caused by the filamentous fungus *Magnaporthe oryzae* (Talbot, 2003). In disease-endemic areas, the yield is generally reduced by 10–20%. Yield reduction reaches 40–50% in severe cases, and grains are not harvested in particularly heavy fields (Dean et al., 2012). Currently, the control of rice blast is mainly based on breeding and utilizing disease-resistant varieties along with supplementation by chemical fungicides. This results in the selection of disease-resistant types and drugs losing their effectiveness. Therefore, developing a new safe, efficient, economical, and convenient way of rice blast prevention and control is of great significance for ensuring food security.

A biological control refers to using living organisms or some metabolically active substances to prevent and control plant diseases, pests, and weeds (Kenneth, 1987). The current biological fungicides are primarily based on microbial fungicides (Fravel, 2005). A microbial fungicide is artificially modified or synthesized using microorganisms or their metabolites or their metabolites as precursors. These biocontrol factors include antagonistic microorganisms, antibiotics, and plant immunity inducers (Qiu et al., 2017). In recent years, the use of microbial fungicides to control rice blast has received increasing attention and has become a research hotspot in rice blast control. *Trichoderma* is a biocontrol fungus with abundant soil resources. It antagonizes various plant pathogens (Woo and Lorito, 2006). *Trichoderma* secretes extracellular enzymes, such as chitinase, cellulase, glucanase, protease, etc., while competing with pathogenic bacteria. These enzymes help dissolve the cell wall of pathogenic fungi, degrade cellulose and glucan in the environment, and acquire more nutrients while inhibiting the growth of pathogenic fungi (Vinalea et al., 2008). A series of studies have shown that *Trichoderma* is very good at inhibiting rice blast with tremendous control (Prabhakaran et al., 2015; Aravindan et al., 2016). In addition, biocontrol proteins like antimicrobial proteins, disease course-related proteins, and trigger proteinswere identified, produced, and applied (Chen M. J. et al., 2014) For example, the distinct antifungal proteins (AFPs) encoded by filamentous fungi exhibited antifungal activity against most fungi tested.

Photosynthetic bacteria are prokaryotic organisms that utilize light as their energy source. They use organic matter, sulfide, ammonia, etc., as hydrogen and carbon sources to perform photosynthesis under anaerobic conditions (Pirson, 1960; Olson, 2010). Photosynthetic bacteria are Gram-negative and belong to the bacterial phylum Eubacteria, order Rhodospirillales. It includes four families, 19 genera, and about 50 species (Olson, 2010). Photosynthetic bacteria are used in many fields due to their wide distribution, green environmental protection, simple production conditions, low cost, significant economic benefits, and convenient use (Idi, 2015). Our previous research identified the controlling effect exerted by the photosynthetic bacterium *Rhodopseudomonas palustris* strain PSB-06 on *M. oryzae*. Moreover, the secreted protein GroEL was significantly antagonistic and pathogenic to *M. oryzae* (Wu et al., 2021).

Currently, chemical control, biological control, and breeding of disease-resistant varieties are the main methods for controlling rice blast, along with cultivation management and other technical means (Jia, 2013). Chemical fungicides are the mainstream choice for rice blast control. Tricyclazole and Isoprothiolane exhibit excellent management of rice blast (Uchida and Fukada, 1983; Shiba and Nagata, 2009). Tricyclazole inhibits the formation of melanin in the rice blast pathogen, thereby inhibiting the germination of conidia and the formation of appressorium of the rice blast pathogen. Subsequently, the invasion of the pathogen is prevented, and the plant is protected. Isoprothiolane acts similarly to organophosphorus fungicides to a certain extent. Its primary mechanism of action is to inhibit the transmethylation of phospholipid choline before synthesis.

Here, the controlling potential of strain PSB-06 was evaluated against *M. oryzae* infection. Further, the combined use of PSB-06 and isopropanethiole or tricyclazole was tested against *M. oryzae* in greenhouse and field trials. The biological agents exerted their antibacterial activity to a greater extent under the action of pesticides. They competed for nutrients required for fungal growth and reduced the use of pesticides to achieve a pollution-free environment to the greatest extent.

Materials and methods

Bacterial strains and culture media

Photosynthetic bacterial PSB-06 and *M. oryzae* strain Guy11 were provided by the Institute of Plant Protection, Hunan Academy of Agricultural Sciences. PSB-06 was grown in the photosynthetic medium (PM) at 30°C under 6500 lx illumination. The *M. oryzae* Guy11 strain was cultured on complete medium (CM) agar plates for 3-7 days at 28°C (Talbot et al., 1993). Guy11 was inoculated onto SDC medium plates for conidiation. The concentration was adjusted to 5×10^4 spores/mL before use.

Sensitivity test of *M. oryzae* to isoprothiolane and tricyclazole

The growth rate assay method was used to identify the sensitivity of *M. oryzae* to isoprothiolane and tricyclazole. Fungi blocks of 4 mm diameter were cut and inoculated into the medium plates with five concentration gradients of 1, 2, 3, 4, and 5 mg/L. The plates were cultivated in the dark at 28°C for seven days to measure the diameter and calculate the relative inhibition rate.

The calculation formula was:

Inhibition rate = (colony diameter of the control group - colony diameter of the treatment group) /colony diameter of the control group × 100%.

Sensitivity test of appressorium formation to isoprothiolane and tricyclazole

For conidiation, 10-day-old conidia were collected with 5 ml of distilled water, filtered through three layers of lens paper and counted with a haemacytometer under a microscope. Conidial germination and appressorium formation were measured on a hydrophobic surface, 30 μL conidia containing 1, 2, 3, 4, and 5 mg/L fungicide, 5‰ (v/v) methanol as control. Appressorium formation rate was counted at 12 h post-inoculation (hpi) under the microscope, more than 200 appressoria were counted for each strain, Photographs were taken at 24 hpi. The experiments were repeated three times.

Effect of isoprothiolane or tricyclazole on PSB-06

After culturing PSB-06 for 5 days, 1 mL of the culture was added to a 120-mL serum bottle containing 100 mL of the photosynthetic medium. Cultures were diluted to 2×10^9 CFU /mL before use (Zhang et al., 2020). To the flasks, isoprothiolane was added to final concentrations of 1, 0.5, and 0.3 mg/L; tricyclazole was added to final concentrations of 2.5, 1.25, and 0.8 mg/L. The mixed cultures were incubated under anaerobic conditions at 30°C and 6,500 lx illumination. After 2, 4, and 6 days, the absorbance of the cultures in the flasks of each treatment group was measured at 600 nm using a microplate spectrophotometer (BioTek Instruments, VT, USA). At 6 days post-infection (6 dpi) (the first day after inoculation), The contents were dispersed on a solid medium plate of photosynthetic bacteria, and the colonies were counted after seven days. The growth of the photosynthetic bacteria under different treatment conditions was determined by the optical density of the culture. The number of colonies was counted to represent the number of viable bacteria. Experiments were independently repeated at least thrice.

Greenhouse experiments

The plant infection test was performed on 4-leaf-sized susceptible rice seedlings from *O. sativa* cultivar CO39 using 5 mL of the conidial suspension. The experimental treatment was divided into six groups: Group 1: Ck; Group 2: 2×10^6 CFU/mL of PSB-06; Group 3: isoprothiolane to achieve a final concentration of 0.3 mg/L; Group 4: tricyclazole to reach a final concentration of 0.8 mg/L; Group 5: 2×10^6 CFU/mL of PSB-06 + isoprothiolane to achieve a final concentration of 0.3 mg/L; Group 6: 2×10^6 CFU/mL of PSB-06 + tricyclazole to reach a final concentration of 0.8 mg/L. The inoculated rice was incubated at 28°C and 90% humidity, cultivated in the dark for 24 h and then transferred back to another moist chamber with a photoperiod of 12 h under fluorescent lights. The disease severity was evaluated at 7 days after inoculation (Chen Y. et al., 2014). Approximately 6 cm long diseased rice blades were photographed to evaluate the virulence of different group.

Field experiments

The field experiment (2021) was conducted in Chunhua (Hunan Province, China). The Nongxiang 42 cultivar rice variety was selected as the control target during the investigation. The soil was cultivated and fertilized according to appropriate agronomic guidelines. The trials included 7 treatment regimens, each with 4 replicates. Each area was 50 square meters. Using the random square method, the following processing method was used: Treatment 1: Control (water); Treatment 2: 2×10^6 CFU/mL of PSB-06; Treatment 3: 6×10^6 CFU/mL of PSB-06; Treatment 4: 1 mg/L of isoprothiolane; Treatment 5: 2.5 mg/L of tricyclazole; Treatment 6: 6×10^6 CFU/mL of PSB-06 + 0.8 mg/L of isoprothiolane; Treatment 7: 6×10^6 CFU/mL of PSB-06 + 2 mg/L of tricyclazole. The treatment was administered using a hand-held sprayer. According to local weather conditions, the fungicide were sprayed three times in a row, every 15 days apart. No other fungicides were applied for controlling rice blast in the experimental field. Experiments were independently repeated at least thrice.

After 15 days of the last spray, 50 rice plants were randomly selected from each experimental area for individual testing. Disease severity and control efficacy were calculated as follows:

Disease severity (%) = [R (number of diseased plants with this index × disease index) /(total number of plants investigated × highest disease index)] × 100.

Control efficacy (%) = [(disease severity of control - disease severity of treated group) /disease severity of control] × 100.

Statistical analysis

Statistical data analysis was carried out using the SPSS software (version 17.0, SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze the biocontrol effects. Student's *t*-test was used to determine any significance between means. $p < 0.01$ indicated statistical significance.

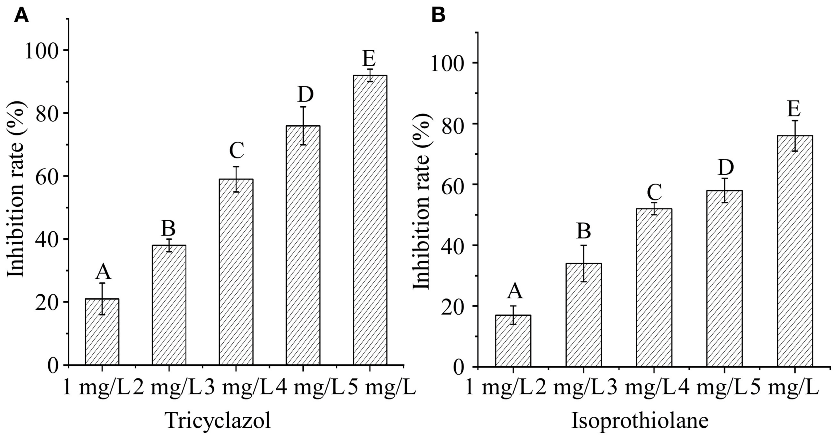

FIGURE 1
Sensitivity test of *M. oryzae* to isoprothiolane and tricyclazole. **(A,B)** The growth inhibition rate is estimated relative to the growth rate of each untreated control. [Inhibition rate = (the diameter of untreated strain − the diameter of treated strain)/(the diameter of untreated strain × 100%)]. Values in columns followed by the same superscript letters indicate no significant difference according to the multiple Duncan test ($P < 0.01$). Three repeats were performed and similar results obtained.

Results

Sensitivity test of *M. oryzae* to isoprothiolane and tricyclazole

The effect of different concentrations of isoprothiolane or tricyclazole was further evaluated on the growth of *M. oryzae*. Our results showed that the growth inhibition rate of tricyclazole and isoprothiolane on *Magnaporthe oryzae* exceeded 50% at the concentration of 3 mg/L (Figure 1A)and 4 mg/L (Figure 1B), respectively, the two fungicides have the same and significant inhibitory effect on the growth of rice blast.

Sensitivity test of appressorium formation to isoprothiolane and tricyclazole

Conidia, play an important role during *M. oryzae* infection, we test the sensitivity of appressorium formation to isoprothiolane and tricyclazole. Under fungicide treatment, the biological morphology of conidia did not not changed (Figure 2A). Then we counted the rate of appressorium formation. Compared with the control group (98%), the rate of appressorium formation was 38 and 59% after treatment with tricyclazole and isoprothiolane at the concentration of 1 mg/l, and the inhibition rate of two fungicides on appressorium formation was the same at the concentration of 5 mg/l (Figures 2B,C). The experimental results showed that the two fungicides had significant inhibition on appressorium formation.

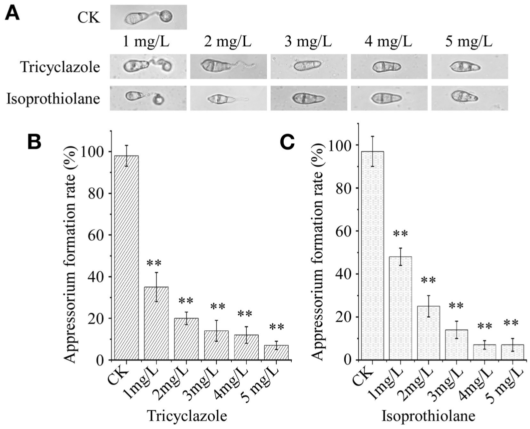

FIGURE 2
Fungicide inhibits appressorium formation. **(A)** Morphological observations of conidia. **(B,C)** Biostatistics of appressorium formation, formation rate = normally formed appressorium / total appressorium × 100% ($p < 0.01$). CK: 5‰ (v/v) methanol.

Effect of isoprothiolane or tricyclazole on strain PSB-06

The photosynthetic bacteria were co-cultured with different concentrations of the two fungicides. The growth of the PSB-06 was not inhibited at isoprothiolane concentrations lower than 0.3 mg/L (Table 1) and tricyclazole concentrations lower than 1.25 mg/L (Table 2). The optical densities (OD_{600}) were 0.749, 1.319, 0.801, and 0.809 at 6 dpi, respectively. Further, the number of CFU of PSB-06 detected on the plate was similar to the optical density result. With increasing concentrations,

TABLE 1 Effect of Tricyclazole on the growth of PSB-06.

Tricyclazole (mg/L)	Absorbance OD (600 nm)			Viable number (× 10^6 CFU/ml)
	2 dpi	4 dpi	6 dpi	
0	0.447 ± 0.019c	0.622 ± 0.018b	0.749 ± 0.015c	121 ± 17c
0.8	0.501 ± 0.012b	1.22 ± 0.01a	1.319 ± 0.013a	186 ± 15a
1.25	0.513 ± 0.009a	0.626 ± 0.017b	0.809 ± 0.016b	152 ± 13b
2.5	0.18 ± 0.012d	0.228 ± 0.011c	0.231 ± 0.013d	62 ± 12d

Each value is the mean (± SE) of at least three replications.
Values in columns followed by the same superscript letters indicate no significant difference according to the multiple Duncan test ($P < 0.05$).

TABLE 2 Effect of Isoprothiolane on the growth of PSB-06.

Isoprothiolane (mg/L)	Absorbance OD (600 nm)			Viable number (× 10^6 CFU/ml)
	2 dpi	4 dpi	6 dpi	
0	0.448 ± 0.011a	0.623 ± 0.012b	0.748 ± 0.009b	133 ± 13a
0.3	0.457 ± 0.008b	0.765 ± 0.011a	0.801 ± 0.007a	139 ± 14a
0.5	0.315 ± 0.011c	0.527 ± 0.013c	0.578 ± 0.012c	108 ± 12b
1	0.295 ± 0.014d	0.425 ± 0.015d	0.475 ± 0.016d	95 ± 15c

Each value is the mean (± SE) of at least three replications.
Values in columns followed by the same superscript letters indicate no significant difference according to the multiple Duncan test ($P < 0.05$).

both fungicides significantly inhibited the growth of PSB-06. At the working concentration of tricyclazole (2.5 mg/L) and isoprothiolane (1 mg/L), the optical densities of the PSB-06 were 0.231 and 0.475 at 6 dpi, respectively.

Plant infection assays

It was interesting to understand the effect of combining PSB-06 with the two fungicides and check the antagonism against rice blast. Hence, different concentrations of the suspensions (PSB-06 and fungicides) were sprayed on 2-week-old rice seedlings. After seven days post-infection, the spores produced tiny and restricted lesions on the rice leaves in the different treatment groups compared with the control group (Figure 3A). Statistical analysis showed that the lesion density (5 cm^2) was only 11 after the combined treatment of isoprothiolane with PSB-06. Moreover, the controlling effect was higher than in other treatment groups (Figure 3B). Thus, the fungicide mixed with PSB-06 exerted better control.

Field experiments

The field test results were consistent with the greenhouse test; each treatment significantly reduced disease occurrence (Table 3). The preventive effect of combining PSB-06 and isoprothiolane or PSB-06 and tricyclazole was higher than that of the individual treatment. The treatment group 5 (2 × 10^6 CFU/mL of PSB-06 + 0.8 mg/L isoprothiolane) was the most efficient and reduced by 76.06%. The effect of group 6 (2 × 10^6 CFU/mL of PSB-06 + 2.5 mg/L tricyclazole) was 72.02%, The least efficient preparation was 2 × 106 CFU/ml PSB-06, resulting in a control efficacy of 47.28%, The effect of high concentration treatment is higher than that of low concentration when applied separately. The controlling effect of the group with a higher concentration (6 × 10^6 CFU/mL) of PSB-06 was 67.99%, and that of the individual fungicides isoprothiolane and tricyclazole was 70.07 and 65.46%, respectively. These results indicate that bactericide and PSB-06 have synergistic bacteriostatic effect, and the synergistic effect of the two fungicides and PSB-06 could control rice blast more effectively.

Discussion

Rice blast causes great harm to agricultural production, and fungicides are the first line of treatment for controlling rice blast. However, fungicides cannot be degraded in time. For example, 4 to 11 distinct fungicidal compounds were detected in 81 samples from vineyards sprayed with fungicides. The ecotoxicological potential of some mixtures in the water stream exceeded the threshold set by the unified principle (Bundschuhab et al., 2016). In Japan, large amounts of pentachloronitrobenzene (PCNB) and major biodegradation products, pentachloroaniline (PCA) and pentachlorothioanisole (PCTA), were detected in agricultural areas (Fushiwaki et al., 1990). Fungicides are

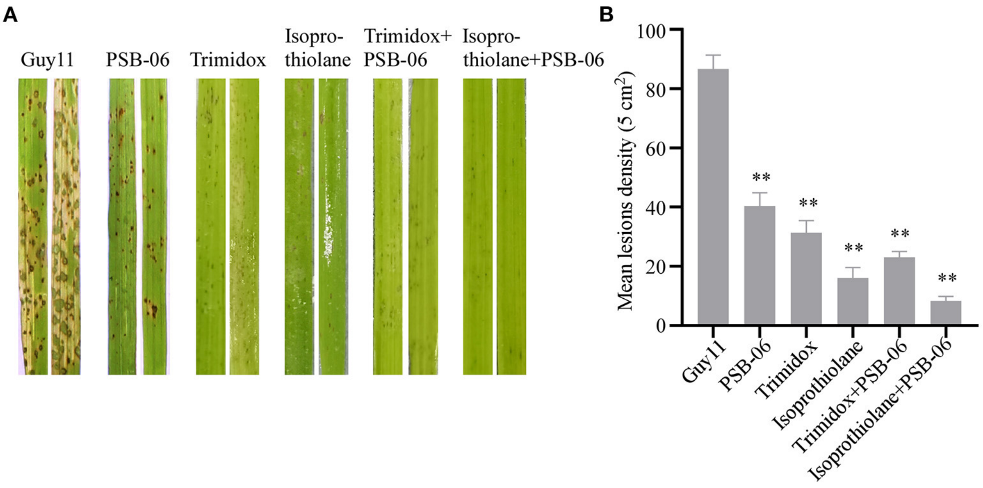

FIGURE 3
Pathogenicity assay under different treatments. **(A)** Leaf spraying assay, four milliliters of conidia suspension (5 × 104 spores/ml) of each strain were sprayed on two-week old rice seedlings. Diseased leaves were photographed at 7 day after inoculation. **(B)** Lesion density was performed by counting lesion numbers of unit area.

TABLE 3 Effect of combinations of PSB-06 and Isoprothiolane or Tricyclazole on controlling rice blast in the field plots.

Treatment	Disease severity (%)	Control efficacy (I) (%)
Control (water)	47.08 ± 11.02A	–
2 × 10^6 CFU/ml PSB-06	24.82 ± 13.03B	47.28 ± 10.67AB
6 × 10^6 CFU/ml PSB-06	15.07 ± 12.48B	67.99 ± 12.07A
Isoprothiolane 1mg /ml	14.09 ± 8.11B	70.07 ± 10.11A
tricyclazole 2.5 mg/ml	16.26 ± 10.22B	65.46 ± 13.33A
2 × 10^6 CFU/ml PSB-06 + 80% isoprothiolane 1 mg/ml	11.27 ± 8.28B	76.06 ± 15.44A
2 × 10^6 CFU/ml PSB-06 + 80% tricyclazole 2.5 mg/ml	13.39 ± 10.15B	72.20 ± 10.28A

Each value is the mean (± SE) of at least three replications.
Values in columns followed by the same superscript letters indicate no significant difference according to the multiple Duncan test ($P < 0.01$).

generally toxic and pose a threat to human health. Fungicidal residues in the environment can contaminate water, soil, and food. Hence, it is essential to choose green control methods.

Biological control is convenient for local production and local application. Biological control measures have a wide range of applications and broad development prospects compared with chemical control. These measures protect and improve farmland ecological environment, do not pollute the environment, reduce the remaining amount of residual poison, and are safe for humans and animals. Moreover, they benefit by delaying the occurrence and development of fungal drug resistance. They also have a continuous and lasting inhibitory effect on some fungi when used constantly. Numerous examples demonstrate the advantages of combining biological and chemical control methods to achieve more effective sterilization and less environmental pollution in the recent upsurge of integrated disease management. Combining azoxystrobin with *Bacillus subtilis* under controlled conditions showed better results against *P. xanthii* than azoxystrobin alone (Gilardi et al., 2008). Two botanical fungicides, Wanis 20 EC and Damet 50 EC, combined with *Pseudomonas fluorescens 1,* reduced the wilt incidence significantly under greenhouse (64%) and field conditions (75%) (Akila et al., 2011). In addition, combined use accelerates the degradation of fungicides. Photosynthetic bacteria significantly degrade organophosphorus fungicides and chemical fungicides containing ester bonds.

In this study, the effects of two fungicides were analyzed on strain PSB-06. Tricyclazole (1.25 mg/L) or isoprothiolane (0.3 mg/L) did not inhibit the growth of strain PSB-06 (Tables 1, 2). Zhang et al. found that photosynthetic bacteria effectively degraded the organophosphorus fungicide methamidophos. The degradation rate of the non-organophosphorus fungicide fenpromethrin reached 67.43% (Zhang et al., 2009a,b, 2014; Kim et al., 2011). Therefore, photosynthetic bacteria were selected as the biological control agent.

As shown as our results, tricyclazole and isoprothiolane have significant inhibitory effect on the growth of *Magnaporthe oryzae* at the concentration of 3 mg/L and 4 mg/L, meanwhile, the concentration of the two fungicides was 5 mg/L, the rate of appressorium germination was only 7%, since conidia play an important role during *M. oryzae* Infection. Hence, they were found suitable for controlling rice blast. However, the present study demonstrated a reduced effect of fungicides in controlling rice blast when used individually. Combining the fungicides with the biocontrol bacterium PSB-06 caused a more significant inhibition of rice blast. The greenhouse and the field experiment results demonstrated a higher control on the rice blast when strain PSB-06 was combined with isoprothiolane compared to their individual treatments. The controlling effect of PSB-06 (6.7×10^6 CFU/mL) on rice blast in the field was 67.99 ±12.07%. This value was lower than that of isoprothiolane (1 mg/mL) (70.07%) and higher than that of tricyclazole (2.5 mg/mL) (65.46%). Co-spraying PSB-06 with a low concentration of isoprothiolane (1 mg/mL) significantly improved the controlling efficiency of rice blast. Thus, to achieve the best biological control, temporary mixing is recommended before spraying. The critical factor for biological control is the efficiency and sustainability by which the agents subsequently inhibit or kill the pathogens; they act on their own or the plant (Kim et al., 2011; Vrieze et al., 2018). The ability to colonize vegetation is key to functioning. PSB-06 grows in a photogenic mode by collecting light energy and separating carbon from carbon dioxide. So, it can adapt to the complex natural field environment with its advantages (Liu et al., 2016).

Our previous study identified a significant increment in the contents of SOD, POD, and chitin after rice was treated with the photosynthetic bacterium. They played an essential role in plant stress resistance (Wu et al., 2021). Su et al. also found that photosynthetic bacteria colonized the leaf periphery of tobacco, producing two plant hormones, 3-indoleacetic acid (3-IAA) and 5-aminolevulinic acid (5-ALA), which promoted plant growth and development (Su et al., 2015, 2017). The controlling efficiency of the current experiment was remarkable. The combination of the antibacterial mechanism of the fungicide and PSB-06 strain improved the controlling effect of rice blast disease.

Combining chemical and biological control is promising. Biological agents exert their antibacterial activity to a greater extent under the action of fungicides. The photosynthetic bacterium PSB-06 was more effective when combined with two fungicides and had better application. It effectively controlled rice blast and reduced the application amount of chemical pesticides to achieve the harmony of green chemistry, high efficiency, and environmental protection.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

YC, DZ, and YL conceived the project. YC, XW, CC, QH, CL, XZ, and XT prepared the samples and performed the research and analyzed the data. YC and XW were major contributor in writing the manuscript. DZ and YL revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Wen Xie,
Institute of Vegetables and Flowers (CASS), China

REVIEWED BY
Yuqian Feng,
Chinese Academy of Forestry, China
Gang Wugang,
Huazhong Agricultural University, China

*CORRESPONDENCE
Yongsheng Zhang
yshzhang@hunau.edu.cn

†These authors have contributed equally to this work

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Morphological characteristics and biological cycle of the hoverfly *Eristalinus arvorum* (Fabricius, 1787) (Diptera, Syrphidae)

Liang Cao[1†], Qing Zeng[2†], Qiuxia Ren[1], Aiping Zeng[1] and Yongsheng Zhang[1*]

[1]College of Plant Protection, Hunan Agricultural University, Changsha, China, [2]Hunan Agricultural Biotechnology Research Institute, Changsha, China

The hoverfly *Eristalinus arvorum* (Fabricius, 1787) (Diptera: Syrphidae), which belongs to the tribe Eristalini, is well known as a pollinating agent for crops and flowering plants in agricultural and natural ecosystems. Large quantity, wide distribution and their ecological function of the hoverfly *E. arvorum* make them an appropriate candidate for use as pollinators and environmental indicator species. However, little information has been known on the morphology and the biological cycle of the hoverfly. In this study, feeding experiments under artificial climate chamber and morphological qualitative and quantitative observations were carried out to study the morphology and the biological cycle of the hoverfly *E. arvorum*. The morphology of eggs, larvae, pupae, and adults of *E. arvorum* were described in detail for the first time. A complete generation of *E. arvorum* lasts about 30.12 ± 0.14 days, and there is no significant difference in body length between males (10.27 ± 0.29 mm) and females (11.16 ± 0.45 mm). The most noteworthy morphological features of *E. arvorum* are the stripes on the compound eyes, the mesonotum, and the abdomen of adults, the chorionic structure consisting of fusiform units on the egg's surface, and the anterior spiracles and pupal spiracles of the pupae. Thus, the detailed description based on morphology and life history will provide the basis for the identification, biodiversity conservation and artificial breeding of the hoverfly *E. arvorum*.

KEYWORDS

pollinating insect, *Eristalinus arvorum*, hoverfly, morphology, electron micrograph

Introduction

Hoverflies belong to the family Syrphidae (Insecta: Diptera) and are one of the most diverse groups in Diptera. At present, there are more than 9,600 known species of hoverflies in the world (Montoya et al., 2012; Zhou et al., 2021), with China being home to 800 of those species from 200 different genera, accounting for one-tenth of the world's species. The life habits of hoverfly larvae are complex and can be divided into predatory, phytophagous, and saprophagous hoverflies based on their feeding habits (Heiss, 1938; Reemer, 2013). The larvae of predatory hoverflies are important natural enemies and can prey on aphids, scale insects, and plant hoppers (Sarthou et al., 2005; Rijn et al., 2013). The larvae of saprophagous hoverflies are the decomposers of ecosystems, feeding on decaying animals and plants, animal feces, and other organic material (Hodson, 1932; Ricarte et al., 2011; Mielczarek et al., 2016; Campoy et al., 2020a). Some of the larvae of phytophagous hoverflies are pests and feed on the roots, stems, and leaves of plants (Creager and Spruijt, 1935; Ricarte et al., 2008, 2017; Nunes-Silva et al., 2010; Dumbardon-Martial, 2016).

Most adult hoverflies visit flowers, feeding on pollen and nectar to supplement nutrition and complete sexual development (Grković et al., 2015; Djellab et al., 2019), they are well-known pollinators of crops, ornamentals, and wild angiosperms (Klecka et al., 2018). Hoverflies are second only to bees as pollinators and even better than bees for some plants (Gladis, 1996). Sánchez et al. (2022) found that pollination by hoverflies could improve the yield and fruit quality of mango under protected cultivation (Sánchez et al., 2022). Ollerton et al. (2012) found that hoverflies play a greater role in pollination than western honeybees. For some plants, hoverflies are the only pollinators; for example, only two species of hoverflies have been found to pollinate the Paphiopedidae (Bänziger, 2002).

Not only do the adults of saprophagous hoverflies visit a variety of flowers, but their larvae can also serve as pollution indicators (Burgio and Sommaggio, 2007). The larvae appear in places with a lot of bacteria in the water and can remove decaying organic material that pollutes the environment (Sommaggio and Burgio, 2014; Moquet et al., 2018; Dunn et al., 2020). Saprophagous hoverflies prey on ∼45% of all known aphids in China. *Eristalinus arvorum* belongs to *Eristalinus* Rondani 1945, the subfamily *Eristalinae*, and is one of the dominant species of hoverflies in southern China. The adults of this species visit a wide range of flowers (Layek et al., 2022) and are commonly found on flowers of vegetable and cash crops such as Brassicaceae, Asteraceae, Labiatae, Rosaceae, Umbelliferae, Leguminosae, and Liliaceae (Van de Weyer and Dils, 1999; Dousti and Hayat, 2006; Almohamad et al., 2009). However, limited research has been conducted on *E. arvorum*. The main objective of this research was to study the morphological characteristics and the biological cycle of *E. arvorum* to improve the artificial rearing and crop pollination applications of this species.

Materials and methods

Sample collection

Adults of *E. arvorum* were collected in the field and kept in a bug dorm (30 cm × 30 cm × 30 cm) with tea pollen and 20% honey water for feeding and soaked grains for laying eggs. Each egg was placed separately in a Petri dish with artificial diets for hatching. When the larvae reached their third instar, they were put into a container with a layer of sawdust for pupation. Pupae were isolated in individual Petri dishes and inspected daily until the emergence of adults. Rearing of *E. arvorum* was performed in a growth chamber at 25 ± 1°C, 70 ± 5% RH, and a constant photoregime of 12L:12D. A total of 120 eggs were selected and

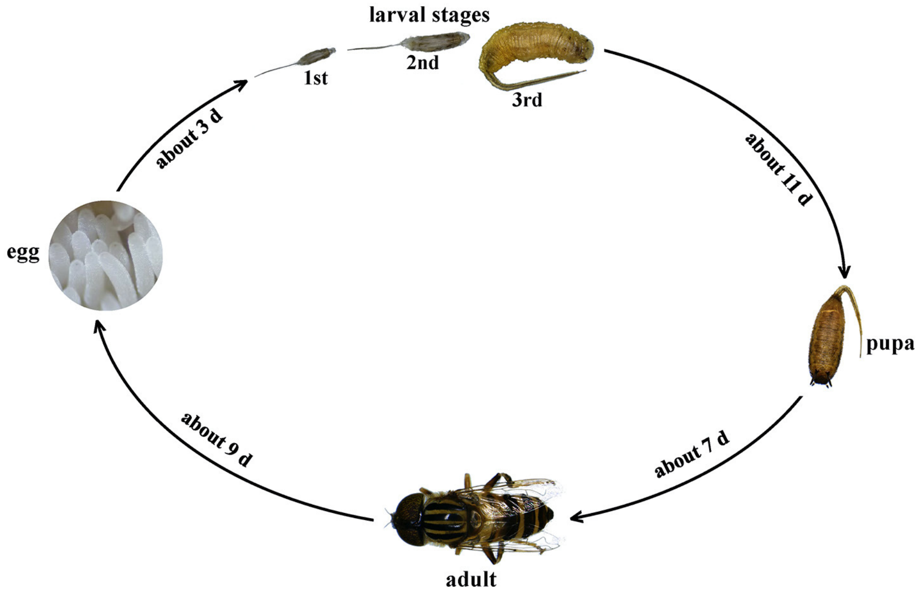

FIGURE 1
Life history of *Eristalinus arvorum*.

raised well into adulthood, and their life histories were observed and recorded daily.

Third-instar larvae were selected as samples for preservation. For permanent preservation, larvae were immersed in cold water to extend them and then heated slowly for about 4 min to kill them. After this, they were preserved in 70% alcohol (Pérez-Bañón et al., 2013; Campoy et al., 2020b). Descriptions were based on preserved specimens, with larval characters checked against living specimens to minimize errors due to preservation (Pérez-Bañón et al., 2013).

Morphological image

Samples of the hoverfly *E. arvorum* from eggs, the first-instar, second-instar, and third-instar larvae, pupae, and adults were collected during the rearing process and then killed cryogenically. The samples were fixed on the sample stage with the double-sided conductive tape of the scanning electron microscope (SEM) and then sputtered with a thin layer of gold (Pérez-Bañón et al., 2013; Campoy et al., 2020b). Once the samples were prepared for observation, they were transferred to the SEM. Secondary electron images were observed and recorded by an SEM-6380LV scanning electron microscope. Eggs, pupal spiracles, and anterior spiracles with a low water content were studied by SEM and others using a stereomicroscope with an imaging system.

Statistical analysis

Twenty samples of male and female adults were measured using the microscopic image measurement software Digimizer 3.2. SPSS 19.0 software was used for conducting the statistical analysis, and the independent samples *t*-test was used to analyze the difference in the bodies of male and female adults.

Results

Biological habits

TABLE 1 The adult morphological data of *Eristalinus arvorum*.

Structure measurement		Female	Male
Prosoma	width (mm)	4.10 ± 0.11	4.10 ± 0.10
	length (mm)	2.42 ± 0.13	2.40 ± 0.12
Antenna	scape (mm)	0.15 ± 0.01**	0.12 ± 0.01
	pedicel (mm)	0.21 ± 0.01	0.22 ± 0.01
	length of flagellum (mm)	0.51 ± 0.04	0.52 ± 0.03
	width of flagellum (mm)	0.34 ± 0.03	0.35 ± 0.03
	length of arista (mm)	1.50 ± 0.14	1.55 ± 0.14
	full length (mm)	0.87 ± 0.07*	0.89 ± 0.05
Mesonotum	width (mm)	3.80 ± 0.13	3.80 ± 0.08
	length (mm)	3.37 ± 0.09	3.20 ± 0.09
Scutellum	width (mm)	2.24 ± 0.07	2.40 ± 0.05
	length (mm)	1.12 ± 0.03	1.15 ± 0.02
Membranous wing	length (mm)	8.32 ± 0.09	8.16 ± 0.07
Wingspan	length (mm)	20.63 ± 0.39	18.84 ± 1.15
Halter	length (mm)	1.01 ± 0.05*	0.97 ± 0.02
Foreleg	coxa (mm)	0.71 ± 0.03	0.70 ± 0.02
	trochanter (mm)	0.48 ± 0.02	0.51 ± 0.09
	femur (mm)	2.33 ± 0.11*	2.06 ± 0.06
	tibia (mm)	2.14 ± 0.10	2.31 ± 0.47
	tarsus (mm)	1.33 ± 0.07	1.13 ± 0.05
	full length (mm)	7.03 ± 0.33*	6.16 ± 0.16
Midleg	coxa (mm)	0.40 ± 0.02	0.34 ± 0.02
	trochanter (mm)	0.46 ± 0.02	0.42 ± 0.02
	femur (mm)	2.48 ± 0.07*	2.38 ± 0.03
	tibia (mm)	2.35 ± 0.07*	2.25 ± 0.02
	tarsus (mm)	1.84 ± 0.08	1.81 ± 0.07
	full length (mm)	7.12 ± 0.21	7.10 ± 0.12
Hindleg	coxa (mm)	0.51 ± 0.02	0.43 ± 0.02
	trochanter (mm)	0.56 ± 0.02	0.52 ± 0.02
	femur (mm)	3.49 ± 0.05	3.27 ± 0.05
	tibia (mm)	2.78 ± 0.04	2.68 ± 0.07
	tarsus (mm)	3.12 ± 0.05	2.83 ± 0.05
	full length (mm)	10.39 ± 0.13*	9.72 ± 0.16
Thorax	length (mm)	4.04 ± 0.33	4.12 ± 0.19
Abdomen	length (mm)	6.00 ± 0.17	5.29 ± 0.10
Body	length (mm)	11.16 ± 0.45	10.27 ± 0.29
	width (mm)	4.18 ± 0.07	4.14 ± 0.05

20 male and 20 female adults were measured. Values are given as mean ± SE. *Significant difference between female and male by the t-test ($P < 0.05$); **Significant difference between female and male by the t-test ($P < 0.01$).

The individual development of *E. arvorum* goes through four stages: eggs, larvae, pupae, and adults, and the larval stage goes through three instars. *E. arvorum* can be reared in captivity, and a complete generation lasts about 30.12 ± 0.14 days (Figure 1). The life history varies with temperature. At a temperature of 25°C, the egg stage lasts about 3.24 ± 0.05 days, and the larval stage lasts about 11.38 ± 0.33 days. It takes about 3.53 ± 0.16 days from the first-st to the second-instar larvae, about 4.12 ± 0.02 days from the second- to the third-instar larva, and about 4.28 ± 0.03 days from the third-instar larva to pupal stage. The pupal stage lasts about 7.24 ± 0.13 days. Adult development to sexual maturity takes about 9.38 ± 0.02 days. Larvae can feed on rotten grains, and pupae can pupate on sawdust. Adults feed on nectars and pollens to complete their development and reproduction, enjoy the sun, and live

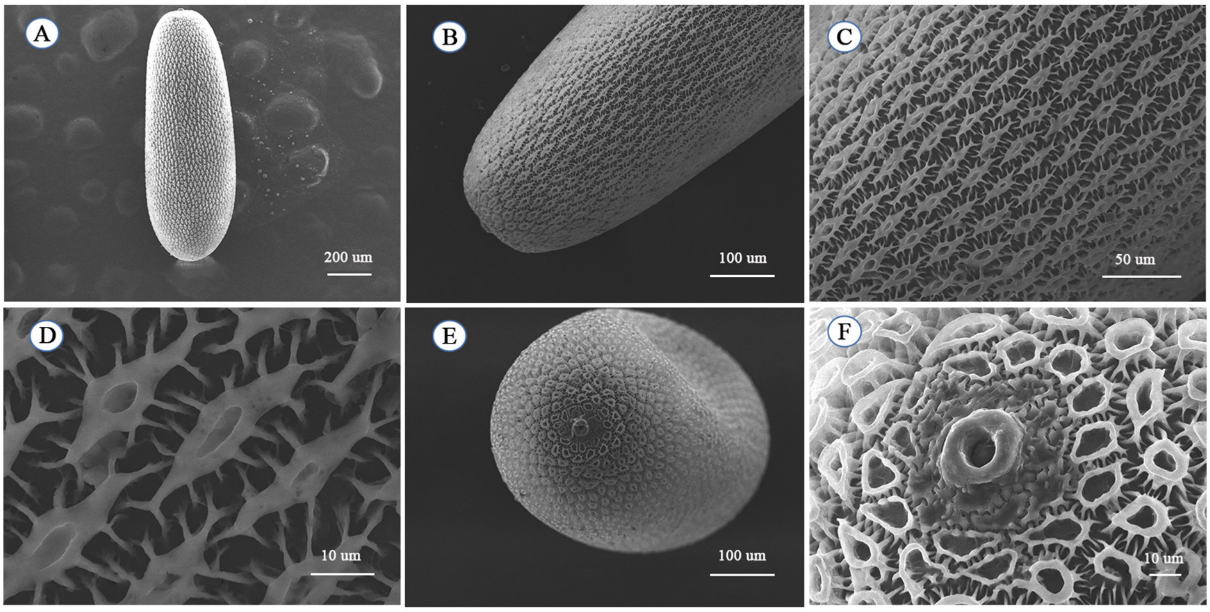

FIGURE 2
Egg of *Eristalinus arvorum*: **(A)** front view of the egg, **(B)** the thinner end of the egg, **(C)** egg reticulation, **(D)** egg reticulation, **(E)** egg top view, **(F)** egg hole.

for about 1 to 2 months. The female adult lays 100–150 eggs at a time.

Adult body data

The adult body of *E. arvorum* is divided into three parts: the prosoma, the mesosoma, and the abdomen. The prosoma bears a pair of antennae, which are divided into the scape, the pedicel, and the flagellum from base to end. The scape in females is significantly longer than that in males (Table 1, $P < 0.01$), but the overall length of the antenna is shorter in females than in males (Table 1, $P < 0.05$). The mesosoma bears two pairs of wings and three pairs of legs. The foreleg and hindleg are significantly longer in females than in males (Table 1, $P < 0.05$). The body length of females is slightly longer than that of males, although there is no significant difference (Table 1).

Description of the morphological characteristics

Egg

The mean lengths of eggs are 1.2 ± 0.12 mm, and the mean maximum widths are 0.35 ± 0.04 mm. Each female adult can lay dozens to hundreds of eggs at a time, depending on the nutritional conditions. Eggs are white when recently laid, become light gray before hatching, and become elongated and rounded at both extremes (Figures 2A,B). The chorionic structure comprises fusiform units, which are branched and deeply hollowed in the middle (Figures 2C,D). The ventral surface is slightly flattened, whereas the dorsal side is convex (Figure 2E). There is a small bulge at the thinner end of the fertilization hole (Figure 2F).

Larva

The larvae are called "rat-tailed maggots" because they have a very long anal segment and a telescopic breathing tube that can extend to the surface of the water. The body is roughly cylindrical with a sub-cylindrical cross-section. The body color is white in the early stage and becomes gray-brown in the later stage. The dorsal and ventral surfaces are slightly flat. The front end is blunt, and the rear end is tapered. The ventral surface is flat and covered with short spicules, while the dorsal side is slightly convex and fully covered with long pubescence backward. The cuticle appears milky-light gray and is slightly translucent when the larva is alive. The first instar larvae (Figures 3A,B) and the second instar larvae (Figures 3C,D) were similar to the third instar larvae (Figures 3E,F) in general morphology. Excluding the posterior trachea, the body lengths of the first instar larvae are 1.51 ± 0.04 to 7.02 ± 0.33 mm (Figures 3A,B), the second instar larvae are 7.02 ± 0.33 to 11.27 ± 0.18 mm (Figures 3C,D) and the third instar larvae are 11.27 ± 0.18 to 23.21 ± 0.07 mm (Figures 3E,F). The body length may vary slightly depending on factors such as environment and

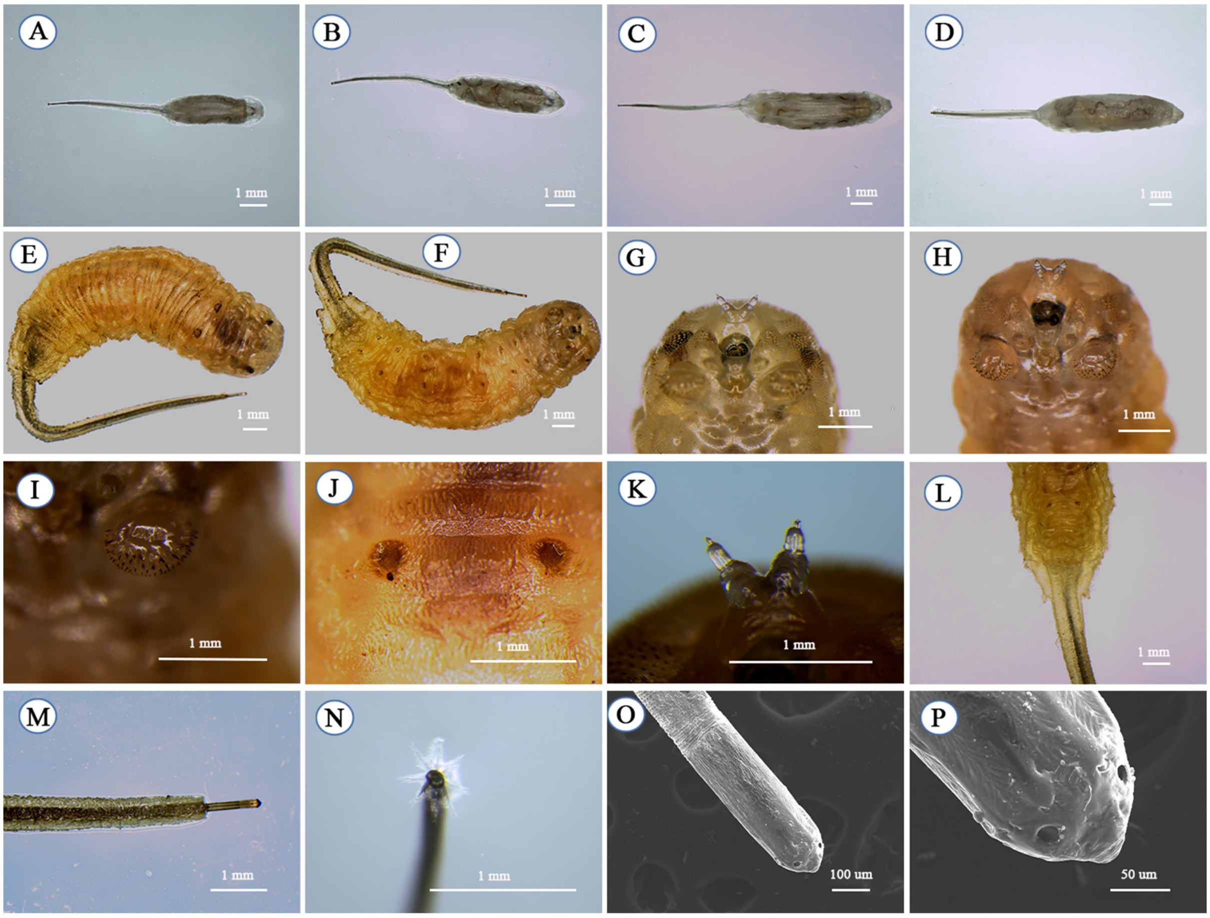

FIGURE 3
Larva of *Eristalinus arvorum*; first-instar larvae: **(A)** dorsal view and **(B)** ventral view; second-instar larvae: **(C)** dorsal view and **(D)** ventral view; third-instar larvae: **(E)** dorsal view, **(F)** ventral view, **(G)** frontal view, **(H)** frontal view, **(I)** midthoracic gastropod, **(J)** pupal respiratory horn foramen, **(K)** antennomaxilary organs, **(L)** the anus, **(M)** posterior tracheal canal, **(N)** posterior trachea plate tip feathers, **(O)** posterior valve tip, and **(P)** fissure.

food. The body surface has many wrinkled rings, and the somite is not obvious (Figures 3E,F).

The third-instar larvae have stout, curved abdominal legs in the mesothorax and 1–6 abdominal segments with toe grooves (Figures 3G–I). Two dots are visible on the back (Figure 3J), where two pupal respiratory horns grow during the pupal stage. The anal process is prominent, and the long digital process retracts into the anus (Figure 3L). A pair of antennomaxilary organs are at the top of the head (Figure 3K). The rear valve is located on the back of the distal segment and attached to a single seat (Figures 3L,M), a telescopic breathing tube extending several times the larva's body length (Figure 3M). Eight feather-like hairs on the end of the rear valve help the rear valve float on the water to breathe while the larva is feeding underwater (Figure 3N). The two plates of the rear valve are closely connected to form a single oval plate with four slits arranged in parallel (Figures 3O,P).

Pupa

The pupa is similar in shape to the larva. The body is pale gray and in the shape of a droplet with a "little tail" at the end of the abdomen that is slightly tan (Figures 4A,B). There is a pair of anterior spiracles at the front of the head and a pair of pupal respiratory horns at the back (Figure 4A). The first to sixth abdominal segments have a pair of abdominal legs (Figure 4B) but they are not prominent. There were no pupal respiratory horns at the early stage of pupation, and the anterior stomata were not obvious. At the later stage of pupation, a pair of pupal spiracles grew (Figures 4C,D) from the foramen of the respiratory horns. The alveolar opening is growing in the front of the pupal spiracles (Figure 4E). There are alveolar openings on the front of the pupal spiracles, varying in number from 4 to 8 (Figures 4F–L). The pupal spiracles folds are covered with villi in a layered distribution (Figure 4M), and there are small irregular bulges at the base with hairs on them (Figures 4N,O). There

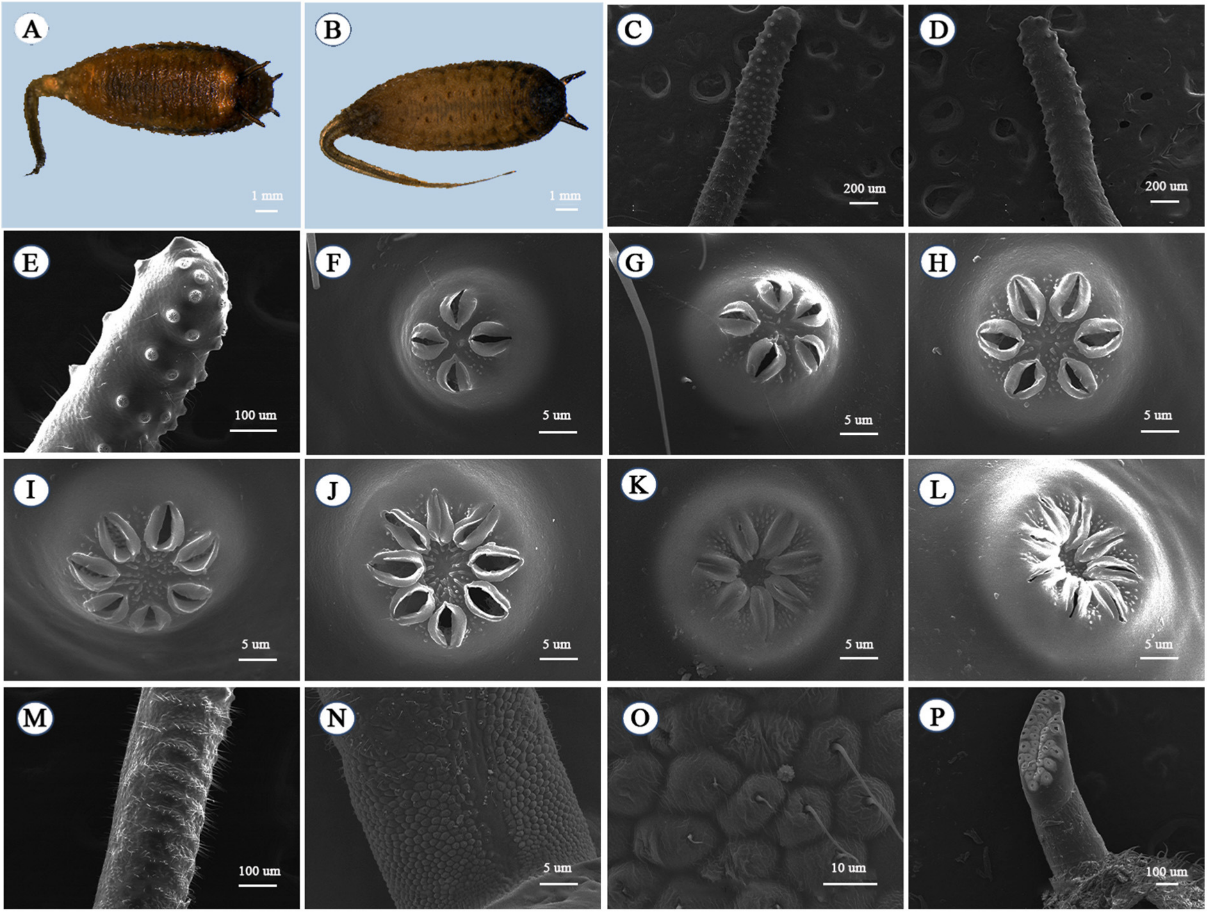

FIGURE 4
Pupa of *Eristalinus arvorum*: **(A)** dorsal view and **(B)** pupa ventral view; pupal spiracle: **(C)** frontal view, **(D)** back view, and **(E)** partial frontal view; spiracular openings: **(F)** four-opening, **(G)** five-opening, **(H)** six-opening, **(I)** seven-opening, **(J)** eight-opening, **(K)** seven closed-mouth, and **(L)** eight closed-mouth; **(M)** pupal spiracle projection base; **(N)** ornamentation on basal part; **(O)** ornamentation on basal part; and **(P)** anterior spiracle.

are breathing openings on the alveolar plates of the anterior spiracles (Figure 4P).

Adult

The compound eyes with different-sized dark spots are dichoptic in females (Figures 5A,E) and holoptic in males (Figures 5C,D). The upper part of the compound eye has dense dark brown short fluff (Figures 5A,C), and the lower part is nearly naked (Figures 5B,D). The vertical bristle is black (Figure 5E). The front is extremely short and slightly convex, covered with grayish-yellow or yellow fluffs and long black hairs (Figures 5E,G). At the top of the head are three ocelli arranged in a triangle (Figures 5E,G). Adults have licking mouthparts (Figures 5F,H). In males, the length between compound eyes is about 1.5 times the length of the triangle on top of the head (Figure 5G). The orange antennae are composed of three segments; the first and second are short, and the third section is oval (Figure 5I).

The mesonotum is bright black with yellow fluff. On the mesonotum, five yellow-gray stripes extend from the front end to the posterior end, with a thin one in the middle and a transverse stripe at the posterior end. The five yellow-gray stripes are thinner in females than in males (Figures 5A,C). The scutellum is pale yellow or bright brown, sometimes with a metallic gloss, with shorter black hair in the middle and long yellow hair on the sides and the ends (Figures 5A,C). A pair of wings are attached to the mesonotum, and the wing membrane of the forewings is transparent (Figure 5J); the hindwings degenerate into halteres (Figure 5K), and the calypteres are black and brown. Three pairs of mesothoracic prolegs are usually brown-yellow or brown-red (Figures 5B,C). The tibia ends of the foreleg and the midleg and the tibiae of the hindleg are black except for the most basal part, and the tarsi

FIGURE 5
Adult of *Eristalinus arvorum*: female: **(A)** dorsal view, **(B)** ventral view, **(E)** prosoma frontal view, and **(F)** prosoma dorsal view; male: **(C)** dorsal view, **(D)** ventral view, **(G)** prosoma frontal view, **(H)** prosoma dorsal view, **(I)** antennae, **(J)** wing membrane, **(K)** halter, **(L)** foreleg, **(M)** midleg, **(N)** hindleg, **(O)** ovipositor and gonostylus, and **(P)** gonosaccus and gonostylus.

ends are brown. Microvilli are on the femora, the tibiae, and the tarsi (Figures 5L–N).

The first abdomere of the abdomen is yellowish to reddish yellow with a shiny skin, and there are red or orange square spots almost over the second abdomere. The abdomen in females is longer than in males. There are yellow lateral stripes on the abdominal segments 2–4 and a pair of yellow lateral spots on segment 5 (Figures 5A,C). At the end of the abdomen is an ovipositor and the gonostylus in females (Figure 5O) and a gonosaccus and the gonostylus in males (Figure 5P).

Discussion

The chorionic structure of the eggs of *E. arvorum* fits with the descriptions of other *Eristalinus* species. *E. arvorum*, *Eristalinus taeniops*, *Eristalinus aeneus*, and *Eristalinus punctulatus* present a chorionic structure with branched fusiform units, but these units, in *E. aeneus*, are not sunk in the middle (Zalat and Mahmoud, 2009; Campoy et al., 2020b). In the case of *E. taeniops*, the branches are broader (Zalat and Mahmoud, 2009), although the general pattern is similar and difficult to distinguish from *E. arvorum*. An adult lays 100 to 150 eggs in masses, which is conducive to collection and feeding.

Despite there being about 75 known species of *Eristalinus* (Sonet et al., 2019), only five species have had their larval stage described: *Eristalinus sepulchralis*, *Eristalinus megacephalus*, *E. aeneus*, *E. taeniops*, and *E. punctulatus* (Pérez-Bañón et al., 2003; Zalat and Mahmoud, 2009; Campoy et al., 2020b). Traditionally, the main diagnostic characters of the larval morphology are found in the transverse band of spicules (number of crochets) and the anterior spiracles (length-width proportion of the spiracle and extension of the spiracular plate relative to the total spiracular length) (Pérez-Bañón et al., 2003; Zalat and Mahmoud, 2009; Campoy et al., 2020b). However, Campoy et al. (2020b) concluded that these characteristics are unreliable for diagnosis by closely examining the morphology of the above species. We found these characteristics of *E. arvorum* to be similar and difficult to distinguish from those of the above species.

Regarding the pupal stage, *Eristalinus* species can be separated by the pupal spiracles, following the key provided by Pérez-Bañón et al. (2003). Because there is scant research on the larvae and the pupae of *Eristalinus* species, *Eristalinus* species are usually identified as adults.

The identification of *E. arvorum* mainly focuses on the adult stage. We found that the main diagnostic characteristics of adults are the stripes on their compound eyes, the mesonotum, and the abdomen. Although the pollination efficiency of *E. arvorum* has not been previously assessed, other *Eristalinus* species have been found to be good pollinators with high floral constancy and positive pollination perspectives (Huda et al., 2015; Latif et al., 2019; Campoy et al., 2020b). For example, *E. punctulatus* is an excellent candidate to be artificially reared and tested as a commercial pollinator in Australia, especially under greenhouse conditions (Campoy et al., 2020b). *Eristalinus aeneus* could be effective pollinators of chickpea and mango trees (Huda et al., 2015; Latif et al., 2019; Sánchez et al., 2022). The morphological photographs of adults in Figures 5A–D show that the chest, the abdomen, and the legs of *E. arvorum* are covered with dense hair, which enables it to carry a large amount of pollen and have high pollination efficiency. In the preliminary field research, we also found that this species can carry large amounts of pollen. The adults of this species have a long lifespan and can live for 2–3 months in our laboratory with sufficient time for pollination. Even though there are fewer flowers in the winter, this species can still be harvested from November to January in Hunan Province, China, due to its high tolerance for cold and hunger. The large number of eggs this species lays is conducive to large-scale artificial production. We have been able to artificially rear *E. arvorum* in captivity, although the feeding system still needs to be improved to make it more efficient. In this process, we found that the composition and temperature of the artificial feed are the key factors affecting its growth and development. Further research is needed to determine the best feeding method.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

LC, QZ, and YZ conceived and designed the project. LC and QZ conducted the experiments and wrote the original draft. LC and QR collected the sample. LC, QZ, and AZ analyzed the data. YZ revised and edited the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Linking life table and predation rate for evaluating temperature effects on *Orius strigicollis* for the biological control of *Frankliniella occidentalis*

Xiaoyun Ren[1], Xiaowei Li[1], Jun Huang[1], Zhijun Zhang[1], Muhammad Hafeez[1,2], Jinming Zhang[1], Limin Chen[1], Shuxing Zhou[1], Lisheng Zhang[3]* and Yaobin Lu[1]*

[1]State Key Laboratory for Managing Biotic and Chemical Treats to the Quality and Safety of Agroproducts, Key Laboratory of Biotechnology in Plant Protection, Ministry of Agriculture and Rural Affairs, Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China, [2]State Key Laboratory of Rice Biology, Ministry of Agriculture Key Lab of Molecular Biology of Crop Pathogens and Insects, Institute of Insect Sciences, Zhejiang University, Hangzhou, China, [3]Key Laboratory of Natural Enemy Insects, Ministry of Agriculture and Rural Affairs, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

EDITED BY
Wen Xie,
Insititute of Vegetables and Flowers (CAAS), China

REVIEWED BY
Shihao Zhou,
Hainan University, China
Ning Di,
Beijing Academy of Agriculture and Forestry Science, China

*CORRESPONDENCE
Yaobin Lu
luybcn@163.com
Lisheng Zhang
zhangleesheng@163.com

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Introduction: *Orius* spp. are generalist predators released in horticultural and agricultural systems to control thrips. Understanding the effects of temperature on the development, predation rate, and population dynamics of *Orius* is essential for identifying the optimal timing of *Orius* release for establishing an adequate population to facilitate synchrony with thrips population growth and to prevent thrips outbreaks. The biological control efficiency of natural enemies as well as predator–prey relationships can be precisely described by integrating life table parameters and the predation rate.

Methods: In this study, the demographic features of *Orius strigicollis* fed on 2nd instar nymphs of western flower thrips (WFT), *Frankliniella occidentalis*, were compared at 18.5, 23.5, 27, and 33°C using the TWOSEX-MSChart program. The CONSUME-MSChart program was used to examine predation rates under different temperatures (18.5, 23.5, and 27°C).

Results: The results showed no significant difference in fecundity among those reared at 18.5, 23.5, and 27°C, but fecundity at these temperatures was significantly higher than that at 33°C. The intrinsic rate of increase (r), finite rate of increase (λ), and net reproduction rate (R_0) were the highest at 27°C. The net predation rate (C_0) and transformation rate (Q_p) were significantly higher at 18.5°C ($C_0 = 168.39$ prey/predator, $Q_p = 8.22$) and 23.5°C ($C_0 = 140.49$ prey/predator, $Q_p = 6.03$) than at 27°C ($C_0 = 138.39$ prey/predator, $Q_p = 3.81$); however, the finite predation rate (ω) showed the opposite trend. In addition to temperature, the stage of *O. strigicollis* at release can affect population dynamics.

Discussion: Our study showed that temperature influenced the demographic traits and predation rates of *O. strigicollis*. When planning a release, the stage of *O. strigicollis* and temperature should be taken into account to establish an adequate population for the control of WFT.

KEYWORDS

predator, temperature, release, augmentative biological control, thrips

Introduction

The western flower thrips (WFT), *Frankliniella occidentalis* Pergrande, is a notorious agricultural and horticultural insect pest worldwide; this species harms plants directly through feeding and oviposition and indirectly through the transmission of plant viruses (Reitz et al., 2011, 2020; Gao et al., 2012; Mouden et al., 2017; He et al., 2020; Wu et al., 2021). Damage by WFT may reduce crop quantity and quality, resulting in severe economic losses for farmers (He et al., 2020; Reitz et al., 2020; Avellaneda et al., 2021). The WFT is a typical *r*-strategist insect pest with a small size, cryptic behavior, and a rapid reproduction rate, and these characteristics make WFT develop resistance to chemical pesticides (Reitz et al., 2011, 2020; Gao et al., 2012; Wu et al., 2021). Moreover, long-term and inappropriate use of chemical pesticides often results in food safety problems, pesticide resistance, and negative impacts on non-target species (Bielza, 2008; Gao et al., 2012; Mouden et al., 2017). Ecology-based methods to reduce insect pest injury in agricultural systems are essential to achieve integrated pest management (Lewis et al., 1997; Demirozer et al., 2012; Mouden et al., 2017). Hence, alternative measures to reduce reliance on chemical insecticides, such as the release of biological control agents, have become important (Mouden et al., 2017; Reitz et al., 2020; Wu et al., 2021).

As biological control agents of key pests that infest cultivated crops, insect predators and parasitoids provide valuable ecosystem services (Landis et al., 2000; Lu et al., 2012; Zhao et al., 2017). The periodic release of natural enemies, also known as augmentative biological control (ABC), is commonly used in greenhouse biological control programs (Messelink et al., 2014; van Lenteren et al., 2018). Minute pirate bugs, *Orius* spp., are useful natural enemies that prey on small and soft-bodied insect pests of agricultural and horticultural crops (De Clercq et al., 2014; Bonte et al., 2017; Zhao et al., 2017; Peterson et al., 2018). Feeding on non-prey food, such as pollen, allows *Orius* to survive when prey are scarce or absent, promoting sustainable insect pest control (Hinds and Barbercheck, 2020; Mendoza et al., 2021, 2022). Accordingly, these generalist predators are preferred in ABC programs and are now mass-reared for pest control purposes, particularly thrips control (Mouden et al., 2017; van Lenteren et al., 2018; Hinds and Barbercheck, 2020; Reitz et al., 2020; Mendoza et al., 2021). Indeed, releasing *O. insidiosus* at a 1:40 WFT ratio can nearly eliminate a WFT population within days (Funderburk et al., 2000). Thus, a comprehensive understanding of the characteristics of *Orius* is crucial for the development and promotion of this natural enemy as a method of thrips control.

Temperature is an important environmental factor that influences both the biological and ecological characteristics of natural enemies (Wallner, 1987; Sørensen et al., 2013; Helgadóttir et al., 2017) as well as the effectiveness of biological control (Montserrat et al., 2013; Boukal et al., 2019; Bai et al., 2022). In mass-rearing programs, natural enemies are reared indoors at an optimum and constant temperature to ensure rapid and efficient production, but these conditions considerably differ from those of crop systems in the field or greenhouse (Montserrat et al., 2013; Sørensen et al., 2013; Helgadóttir et al., 2017), in which temperatures change with seasonal oscillations and diel activity rhythms. As the temperature has a direct effect on the survival, development, and reproduction of natural enemies (Helgadóttir et al., 2017), population dynamics will be affected by temperature fluctuations (Barton and Schmitz, 2009; Montserrat et al., 2013). Thus, the development, predatory ability, and population dynamics of natural enemies in response to various temperatures must be systematically assessed; these results will facilitate predictions of the ecological effects of climate change on predator-prey interactions (Boukal et al., 2019).

Numerous studies have assessed the influence of temperature on the development, fecundity, and functional response of *Orius* species (Nagai and Yano, 1999; Ohta, 2001; Baniameri et al., 2005; Ballal et al., 2017). Experimental studies on the integrated life table traits and predatory rates of *Orius* species in response to temperature can precisely elucidate the biological control efficiency of *Orius* and predator-prey relationships (Ding et al., 2021). *Orius strigicollis* is a major natural enemy of pests among agroecosystems in southern China, Korea, and Japan (Musolin et al., 2004; Cho et al., 2005; Musolin and Ito, 2008; Ding et al., 2021) that has been mass-reared for thrips control (van Lenteren, 2012; Tuan et al., 2016; van Lenteren et al., 2018). Releasing this biological control agent and establishing an adequate population promotes synchronization with thrips population growth and prevents thrips outbreaks. In this study, to fully understand the effect of temperature on the population dynamics and development of *O. strigicollis*, the demographic characteristics (including development, reproduction, survival, and longevity) of *O. strigicollis* reared at constant temperatures of 18.5–33°C and fed 2nd instar WFT nymphs were quantitatively described using the life table analysis program (TWOSEX-MSChart, Chi, 2022a), which can precisely describe stage differentiation and correctly evaluate the fitness of populations (Chi et al., 2020). The predation rates of *O. strigicollis* fed WFT under different temperature conditions (18.5–27°C) were also evaluated with the CONSUME-MSChart computer program (Chi, 2022b). Additionally, the population growth of *O. strigicollis* under different temperature conditions was projected using the TIMING-MSChart program (Chi, 2022c).

Materials and methods

Insects

Western flower thrips were collected from *Cucumis melo* L. grown in the greenhouse at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (39.53 °N, 116.70 °E) in Beijing, China, in 2007 and reared on bean pods (*Phaseolus vulgaris* L.) under laboratory conditions at 26±1°C, with relative humidity (RH) of 60 ± 10% and an L14:D10 photoperiod. *Orius strigicollis* were collected from thrips-damaged pepper (*Capsicum annuum* L.) plants grown in the greenhouse at the Yangdu Experiment Station of Zhejiang Academy of Agricultural Sciences (30.53 °N, 120.68 °E) in Jiaxing, Zhejiang Province, China, in 2016 and reared on WFT and bean pods, which were used as a water source and oviposition substrate for *O. strigicollis*. To avoid the negative effects of inbreeding, both WFT and *O. strigicollis* were collected every year from the field to propagate with the laboratory colony.

Life table and predation rate of *O. strigicollis* under various temperature conditions

Orius strigicollis eggs oviposited for 24 h on bean pods were selected and randomly separated into four groups, with 82, 98, 78, and 104 eggs per group. The egg groups were maintained at 18.5, 23.5, 27, and 33°C, respectively, with 60 ± 10% RH and an L16:D8 photoperiod. Every 24 h, the eggs were checked, and for viable eggs, the demographic traits were accurately determined (Mou et al., 2015; Ding et al., 2021). In total, 74 (18.5°C), 88 (23.5°C), 71 (27°C), and 89 (33°C) eggs were used as the initial samples. Newly hatched nymphs were individually transferred to Petri dishes (6 cm in diameter) with a fine brush. Two holes (1 cm in diameter) covered with a fine-mesh screen in the lid of the Petri dish allowed for ventilation. A piece of fresh bean pod (2–3 cm in length) was provided as a water source, and different numbers of 2nd instar WFT were provided as food for *O. strigicollis* nymphs (Ding et al., 2021). To prevent the escape of the WFT from the Petri dish, each dish was sealed with Parafilm. Every 24 h, the developmental stage of the nymphs was observed, and fresh bean pods and WFT were replaced in the Petri dish. Since predatory ability varies with developmental stage in *O. strigicollis*, different numbers of WFT were provided daily: 10 WFT for 1st (N1) and 2nd instar (N2), 15 WFT for 3rd (N3) and 4th instar (N4), and 20 WFT for 5th instar (N5) *O. strigicollis* every day (Ding et al., 2021). The number of WFT provided for *O. strigicollis* every day under various temperature conditions was sufficient. The numbers of WFT consumed by *O. strigicollis* reared at 18.5–27°C were recorded every 24 h.

When the adults emerged, sex was determined. Females and males were paired, and each adult pair was moved into a Petri dish (9 cm in diameter) with a 3 cm-diameter hole on the lid covered with a fine-mesh screen. A fresh bean pod (∼6 cm in length) was used as the oviposition substrate, and a sufficient number of thrips (30 WFT) were provided as prey. Fecundity, longevity, and survival were recorded every 24 h, and fresh bean pods and WFT were provided daily. If the number of females exceeded that of males or a paired male died, additional males of the same age from the laboratory colony were paired with the females, but the longevity and the number of consumed WFT were not recorded. If the number of males exceeded that of females or a paired female died, additional females of the same age from the laboratory colony were paired with the males, but female fecundity, longevity, and the number of consumed WFT were not recorded (Ding et al., 2021). The numbers of WFT consumed by adult *O. strigicollis* reared at 18.5–27°C were recorded, and a male-to-female ratio of 1:2 was used to determine the consumption rate of paired adults (Ding et al., 2021).

Data analysis

Life table analysis

The demographic characteristics of *O. strigicollis* fed on WFT in response to temperature were analyzed using the computer program TWOSEX-MSChart (Chi, 2022a) based on the age-stage, two-sex life table theory (Chi and Liu, 1985; Chi, 1988; Chi et al., 2020). The age-stage-specific survival rate (s_{xj}) (where x indicates age and j indicates stage), age-specific survival rate (l_x), age-specific fecundity (m_x), net maternity ($l_x m_x$), age-stage-specific fecundity (f_{xj}), intrinsic rate of increase (r), net reproduction rate (R_0), finite rate of increase (λ), and mean generation time (T) were calculated according to Chi and Liu (1985). The age-stage life expectancy (e_{xj}) was calculated according to Chi and Su (2006), and the age-stage reproductive value (v_{xj}) was calculated according to Tuan et al. (2014a,b). R_0 was calculated as follows:

$$R_0 = \sum_{x=0}^{\infty} l_x m_x \tag{1}$$

The interactive bisection method and the Euler-Lotka equation (Goodman, 1982) were used to estimate r:

$$\sum_{x=0}^{\infty} e^{-r(x+1)} l_x m_x = 1 \tag{2}$$

λ and T were calculated as follows:

$$\lambda = e^r \tag{3}$$

$$T = \frac{ln(R_0)}{r} \tag{4}$$

The variances and standard errors of all of the parameters were estimated with 100,000 resamplings in the TWOSEX-MSChart program. Significant differences in the parameters under different temperature conditions were determined using the paired bootstrap test based on the 95% confidence intervals.

Predation rate analysis

The predation rates of *O. strigicollis* at different temperatures (18.5, 23.5, and 27°C) were calculated according to Chi and Yang (2003) using the CONSUME-MSChart computer program (Chi, 2022b). The age-specific predation rate (k_x, the number of prey consumed by the surviving *O. strigicollis* at age x) was calculated as follows:

$$k_x = \frac{\sum_{j=1}^{\beta} s_{xj} c_{xj}}{\sum_{j=1}^{\beta} s_{xj}} \tag{5}$$

where c_{xj} is the number of 2nd instar nymphs of WFT consumed by *O. strigicollis* at age x and stage j, which was calculated as follows:

$$c_{xj} = \frac{\sum_{i=1}^{n_{xj}} d_{xj,i}}{n_{xj}} \tag{6}$$

where $d_{xj,i}$ is the number of WFT consumed by the ith *O. strigicollis* at age x and stage j, and n_{xj} is the number of *O. strigicollis* surviving to age x and stage j. The age-specific net predation rate (q_x) was calculated as follows:

$$q_x = l_x k_x = \sum_{j=1}^{\beta} s_{xj} c_{xj} \tag{7}$$

The cumulative net predation rate (C_x) (the number of prey consumed per predator from birth to age x) and the net predation rate (C_0) (the total number of prey consumed per predator over its lifetime) were calculated as follows:

$$C_x = \sum_{i=0}^{x} q_i = \sum_{i=0}^{x} l_i k_i \tag{8}$$

$$C_0 = \sum_{i=0}^{\infty} l_x k_x \tag{9}$$

The transformation rate (Q_p) indicates the number of prey consumed to produce one offspring and was calculated as follows:

$$Q_p = \frac{C_0}{R_0} \tag{10}$$

Following Ding et al. (2021), the predation rate of each predator in stage j (P_j) was calculated as follows:

$$P_j = \frac{\sum_{i=1}^{n_j} p_{ij}}{n_j} \tag{11}$$

where p_{ij} is the number of prey killed by predator i in stage j and n_j is the number of predators that survived in stage j.

The daily predation rate per predator in stage j (D_j) (Ding et al., 2021) was calculated as follows:

$$D_j = \frac{\sum_{x=a}^{b} c_{xj} s_{xj}}{\sum_{x=a}^{b} s_{xj}} \tag{12}$$

where a and b indicate the first and last ages of stage j, respectively. The finite predation rate (ω) (Yu J. K., et al., 2013) was calculated as follows:

$$\omega = \lambda \sum_{i=0}^{\infty} \sum_{j=1}^{\beta} a_{xj} c_{xj} \tag{13}$$

where a_{xj} indicates the proportion of individuals at age x and stage j.

Significant differences in the parameters under different temperature conditions were determined using the paired bootstrap test in the TWOSEX-MSChart program (Chi, 2022a).

Population projection

The population growth of *O. strigicollis* under different temperature conditions was projected using the computer program TIMING-MSChart (Chi, 2022c). All of the figures were drawn with GraphPad Prism software (8.0 version).

Results

Life table of *O. strigicollis* under different temperatures

The temperature had a strong effect on the developmental duration of *O. strigicollis*, as the developmental duration shortened with an increase in temperature (Table 1). At 18.5°C, the developmental durations of both *O. strigicollis* eggs and nymphs were the longest, followed by those at 23.5°C and 27°C. At 33°C, the developmental durations of *O. strigicollis* eggs and nymphs were the shortest, indicating a fast development rate (Supplementary Table 1). The adult longevity of *O. strigicollis* was not significantly different between the 23.5 and 27°C treatments; both were significantly lower than that at 18.5°C but longer than that at 33°C.

The age-stage survival rate (s_{xj}) of *O. strigicollis* under the different temperature conditions is shown in Figure 1; obvious overlaps existed among the stages at all of the tested temperatures. The survival rates of both eggs and nymphs varied with temperature; the survival rates were higher at 18.5, 23.5, and 27°C (Figures 1A–C) and lower at 33°C (Figure 1D).

The adult preoviposition period (APOP), total preoviposition period (TPOP), and oviposition days (O_d) were significantly longer at 18.5°C than those at 23.5, 27,

TABLE 1 Developmental times (d) and adult longevities (d) of *Orius strigicollis* fed on *Frankliniella occidentalis* at different temperatures.

Stage	**18.5°C**		**23.5°C**		**27°C**		**33°C**	
	n	**Mean ± (SE)**	***n***	**Mean ± (SE)**	***n***	**Mean ± (SE)**	***n***	**Mean ± (SE)**
Developmental time								
Egg	74	10.27± 0.15 a	88	4.34 ± 0.05 b	71	3.72 ± 0.07 c	89	2.11 ± 0.06 d
N1	66	6.52 ± 0.11 a	81	2.06 ± 0.04 b	65	1.91 ± 0.08 b	75	1.56 ± 0.06 c
N2	60	5.79 ± 0.12 a	73	3.16 ± 0.11 b	60	1.65 ± 0.06 c	68	1.26 ± 0.05 d
N3	58	5.40 ± 0.14 a	73	2.99 ± 0.11 b	58	1.40 ± 0.07 c	66	1.26 ± 0.06 c
N4	57	5.84 ± 0.20 a	69	2.25 ± 0.10 b	56	1.46 ± 0.08 c	64	1.38 ± 0.06 c
N5	55	9.49 ± 0.21 a	66	3.77 ± 0.10 b	54	3.24 ± 0.09 c	57	2.79 ± 0.07 d
Pre-adult	55	43.25 ± 0.36 a	66	18.48 ± 0.18 b	54	13.31 ± 0.16 c	57	10.32 ± 0.14 d
Adult longevity								
All adult	55	42.55 ± 2.40 a	66	16.17 ± 0.95 b	54	14.02 ± 0.87 b	57	4.75 ± 0.32 c
Female adult	26	44.04 ± 3.14 a	32	17.47 ± 1.49 b	33	14.36 ± 1.13 b	30	5.33 ± 0.42 c
Male adult	29	41.21 ± 3.62 a	34	14.94 ± 1.18 b	21	13.48 ± 1.39 b	27	4.11 ± 0.45 c

Different letters within the same rows indicate significantly different temperatures determined by the paired bootstrap test with 100,000 resamplings ($P < 0.05$).

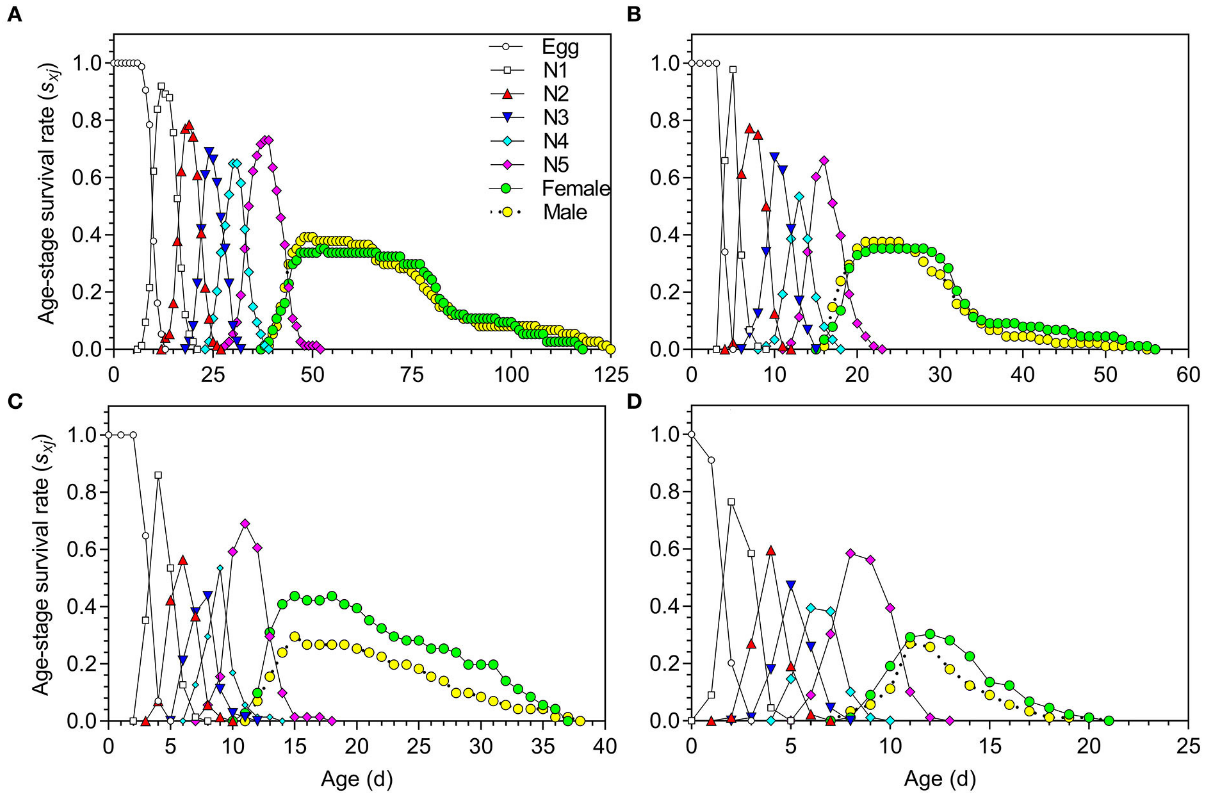

FIGURE 1
Age-stage survival rate (s_{xj}) of *Orius strigicollis* fed on *Frankliniella occidentalis* under 18.5°C **(A)**, 23.5°C **(B)**, 27°C **(C)**, and 33°C **(D)**.

and 33°C (Table 2). The parameters of APOP and O_d did not significantly differ between the 23.5°C (APOP = 4.73 days, O_d = 10.67 days) and 27°C (APOP = 3.60 days, O_d = 9.23 days) treatments, but both were significantly longer than those at 33°C (APOP = 1.78 days, O_d = 3.04 days). The mean fecundity (*F*) of *O. strigicollis* showed no significant difference among the 18.5°C (58.31 eggs/female), 23.5°C (64.03 eggs/female), and 27°C (78.09 eggs/female) treatments but was obviously higher

TABLE 2 Preoviposition period, oviposition period, and fecundity of *Orius strigicollis* preying on *Frankliniella occidentalis* at different temperatures.

Parameters	18.5°C		23.5°C		27°C		33°C	
	n	Mean ± (SE)	*n*	Mean ± (SE)	*n*	Mean ± (SE)	*n*	Mean ± (SE)
Adult preoviposition period, APOP (d)	24	11.79 ± 1.40 a	30	4.73 ± 0.70 b	30	3.60 ± 0.57 b	23	1.78 ± 0.20 c
Total preoviposition period, TPOP (d)	24	55.12 ± 1.45 a	30	23.40 ± 0.68 b	30	16.83 ± 0.63 c	23	11.78 ± 0.30 d
Oviposition days (O_d, d)	24	24.96 ± 2.13 a	30	10.67 ± 1.25 b	30	9.23 ± 0.91 b	23	3.04 ± 0.41 c
Fecundity (*F*, all eggs/female)	26	58.31 ± 6.48 a	32	64.03 ± 8.00 a	33	78.09 ± 9.82 a	30	17.87 ± 3.70 b
Fecundity (*f*, eggs/female/d)	26	1.32 ± 0.14 c	32	3.57 ± 0.35 b	33	5.28 ± 0.50 a	30	2.93 ± 0.52 bc

Different letters within the same rows indicate significantly different temperatures determined by the paired bootstrap test with 100,000 resamplings ($P < 0.05$).

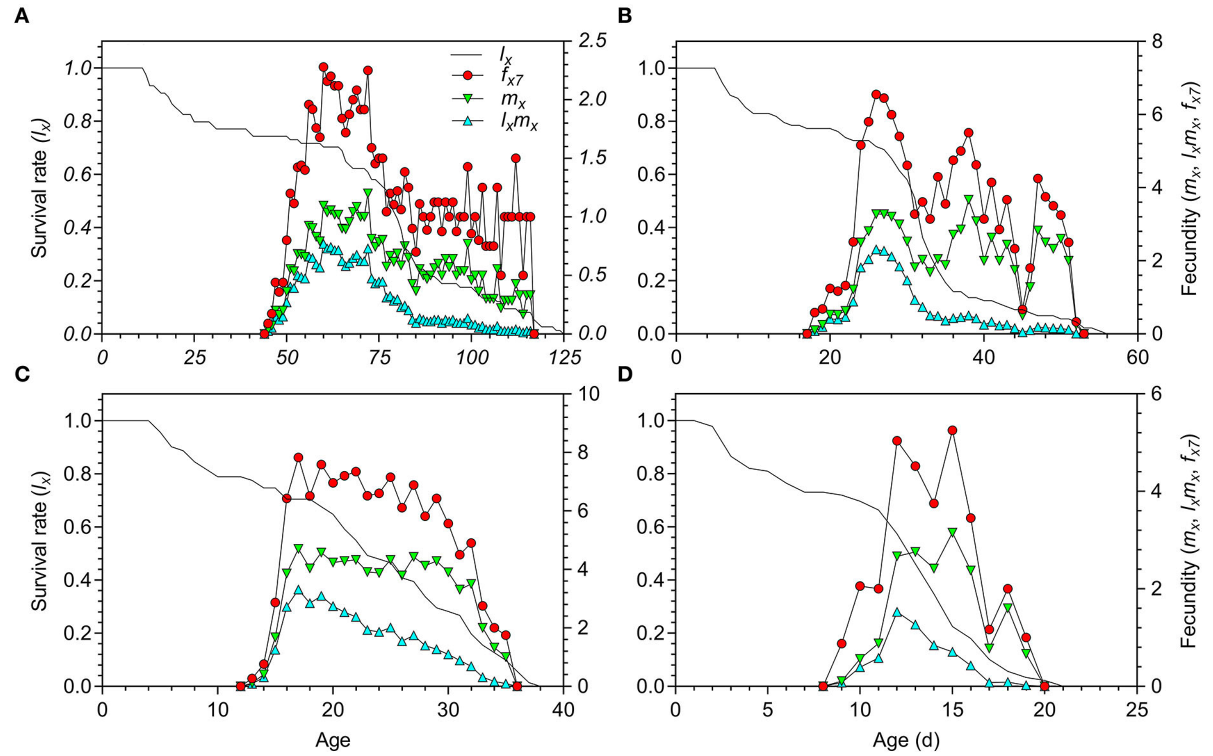

FIGURE 2
Age-specific survival rate (l_x), fecundity (m_x), net maternity (l_xm_x), and female age-specific fecundity (f_{x7}) of *Orius strigicollis* fed on *Frankliniella occidentalis* under 18.5°C **(A)**, 23.5°C **(B)**, 27°C **(C)**, and 33°C **(D)**.

than that at 33°C (17.87 eggs/female). However, the number of eggs laid per female per day (f) at 27°C (5.28 eggs) was obviously larger than those at 23.5°C (3.57 eggs), 33°C (2.93 eggs), and 18.5°C (1.32 eggs). Among the tested temperatures, the female age-specific fecundity (f_{x7}) first increased rapidly, peaked, and then decreased (Figure 2). The peak f_{x7} of *O. strigicollis* was 7.83 eggs/female at 27°C, which was higher than that at 23.5°C (6.55 eggs/female), 33°C (5.25 eggs/female), and 18.5°C (2.28 eggs/female). Fecundity (m_x) and net maternity (l_xm_x) are plotted in Figure 2.

The life expectancy (e_{xj}) of *O. strigicollis* decreased with increasing temperature (Figure 3). The highest reproductive values (v_{xj}) of *O. strigicollis* at 18.5, 23.5, 27, and 33°C were 29.43, 34.60, 35.78, and 13.57 day^{-1}, respectively (Figure 4).

The intrinsic rate of increase (r) and finite rate of increase (λ) of *O. strigicollis* were highest at 27°C ($r = 0.1682$ day^{-1}, $\lambda = 1.1833$ day^{-1}) but did not differ significantly from those of at 33°C ($r = 0.1298$ day^{-1}, $\lambda = 1.1387$ day^{-1}) (Table 3); those at 23.5°C ($r = 0.1094$ day^{-1}, $\lambda = 1.1157$ day^{-1}) and 18.5°C ($r = 0.0457$ day^{-1}, $\lambda = 1.0467$ day^{-1}) were lower.

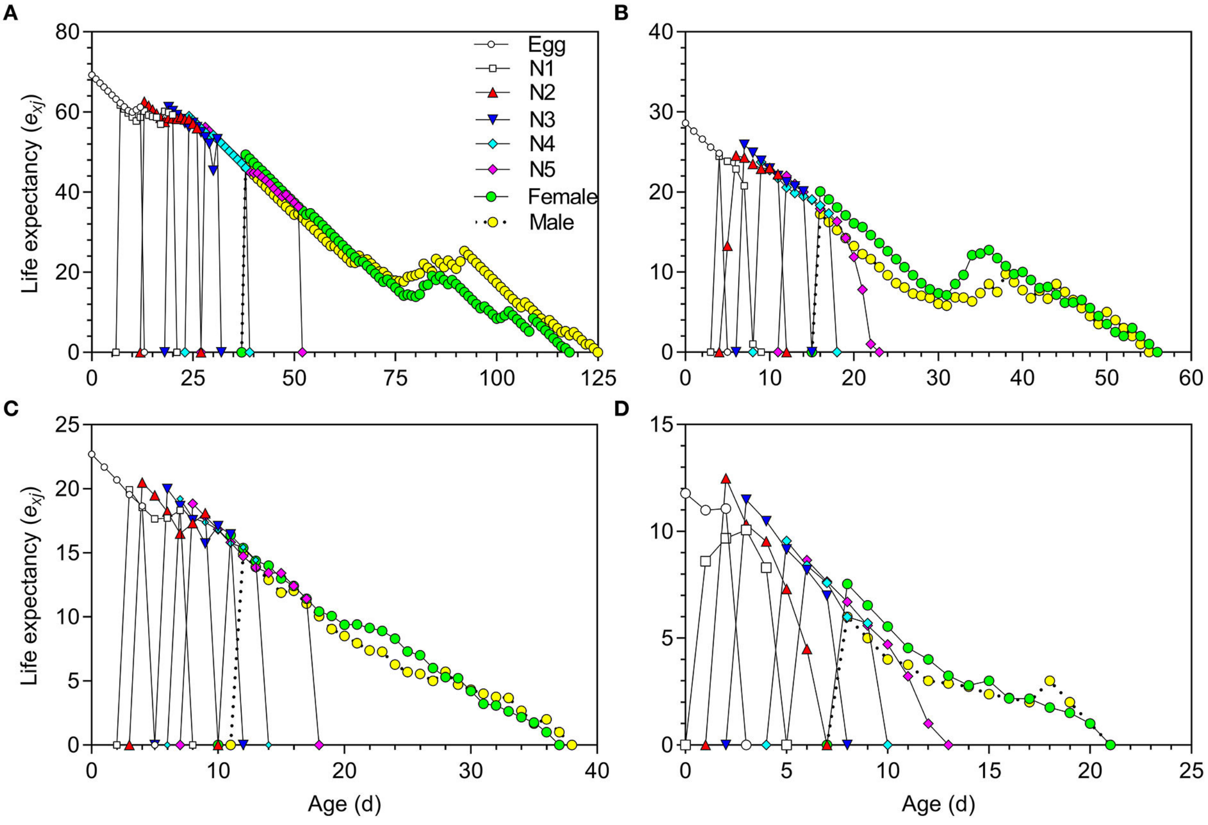

FIGURE 3
Life expectancy (e_{xj}) of *Orius strigicollis* fed on *Frankliniella occidentalis* at 18.5°C **(A)**, 23.5°C **(B)**, 27°C **(C)**, and 33°C **(D)**.

The net reproductive rate (R_0) of *O. strigicollis* at 27°C (36.29 offspring/individual) was not significantly different from that at 23.5°C (23.28 offspring/individual), but the R_0 values at both of these temperatures were significantly higher than those at 18.5°C (20.48 offspring/individual) and 33°C (6.02 offspring/individual). The mean generation time (T) of *O. strigicollis* decreased as the temperature increased. The longest generation time was observed at 18.5°C (66.06 days), while the shortest generation time was observed at 33°C (13.83 days).

Predation rate

The daily predation rate (D_j) of *O. strigicollis* increased as the temperature increased within the tested temperature range (Table 4), and the D_j of each stage was the highest at 27°C. For *O. strigicollis* female adults, the D_j showed no significant difference between 27°C (9.88 prey/predator) and 23.5°C (9.44 prey/predator), but these D_j values were significantly higher than that at 18.5°C (4.40 prey/predator). The total numbers of WFT consumed (P_j) during the preadult stage were 69.96, 63.61, and 60.28 per predator at 18.5, 23.5, and 27°C, respectively (Table 5). The adult P_j at 18.5°C was higher than those at 23.5 and 27°C. The P_j of female adults was the highest at 18.5°C (198.87 prey/predator), followed by 23.5°C (164.92 prey/predator) and 27°C (141.93 prey/predator).

The first peaks of the age-specific predation rate (k_x) and age-specific net predation rate (q_x) in *O. strigicollis* fed on WFT at 18.5°C occurred at the age of 39.00 days in the N5 stage; these rates then decreased until adult emergence at the age of 44.00 days (Figure 5A). The peaks occurred significantly later than those at 23.5°C (15.00 days) (Figure 5B) and 27°C (10.00 days) (Figure 5C).

The net predation rate (C_0) varied significantly among temperature conditions (Table 6). Each *O. strigicollis* consumed an average of 168.39 prey over its lifetime when reared at 18.5°C, which was significantly higher than the numbers of prey consumed at 23.5°C (140.49 prey/predator) and 27°C (138.39 prey/predator). The finite predation rate (ω) increased as the temperature increased. The transformation rate (Q_p) indicated that at 18.5°C, *O. strigicollis* needed to consume 8.22 WFTs to produce one egg, which was not significantly different from that at 23.5°C (6.03); however, both were higher than that at 27°C (3.81).

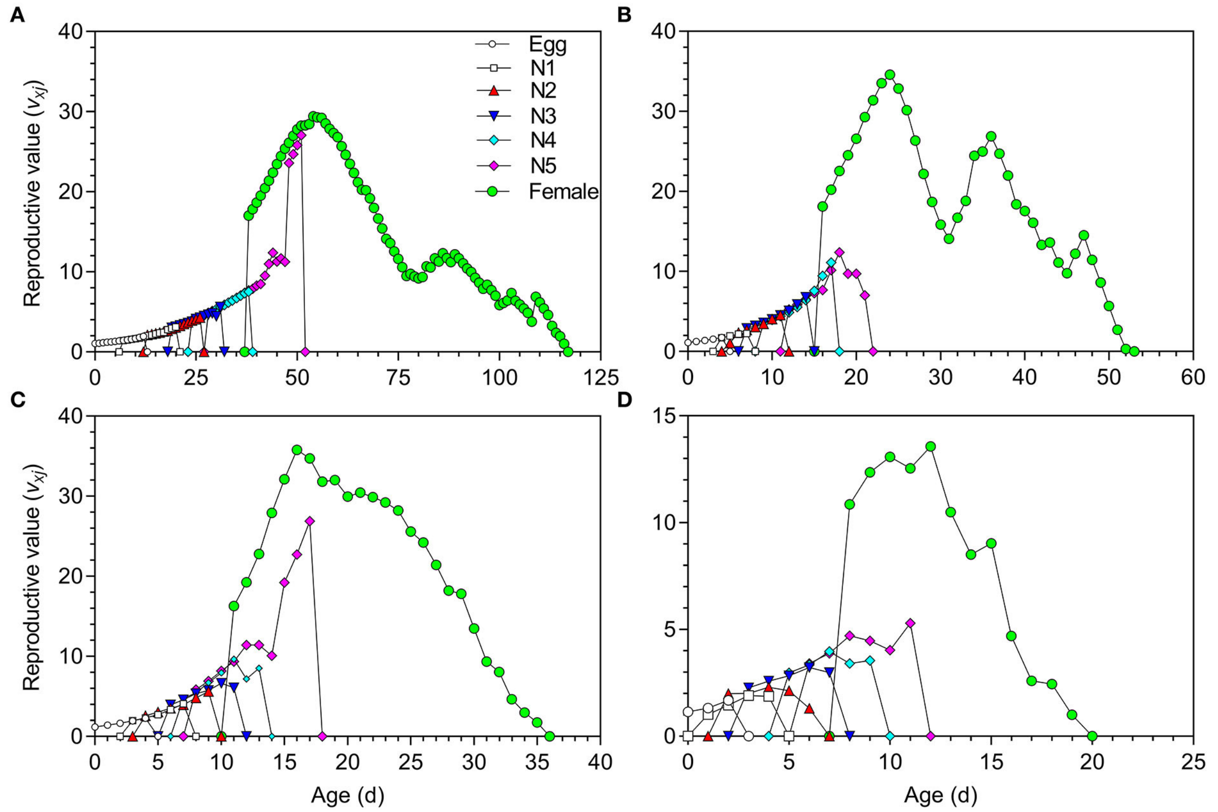

FIGURE 4
Reproductive value (v_{xj}) of *Orius strigicollis* fed on *Frankliniella occidentalis* at 18.5°C **(A)**, 23.5°C **(B)**, 27°C **(C)**, and 33°C **(D)**.

TABLE 3 Population parameters of *Orius strigicollis* preying on *Frankliniella occidentalis* at different temperatures.

Population parameters	18.5°C		23.5°C		27°C		33°C	
	n	Mean ± (SE)	*N*	Mean ± (SE)	*n*	Mean ± (SE)	*n*	Mean ± (SE)
Intrinsic rate of increase, r (d^{-1})	74	0.0457 ± 0.0032 c	88	0.1094 ± 0.0065 b	71	0.1676 ± 0.0085 a	89	0.1298 ± 0.0185 ab
Finite rate of increase, λ (d^{-1})	74	1.0467 ± 0.0034 c	88	1.1157 ± 0.0072 b	71	1.1826 ± 0.0100 a	89	1.1387 ± 0.0209 ab
Net reproductive rate, R_0 (offspring/individual)	74	20.48 ± 3.94 b	88	23.28 ± 4.36 a	71	36.29 ± 6.45 a	89	6.02 ± 1.52 c
Mean generation time, T (d)	74	66.06 ± 1.66 a	88	28.76 ± 0.74 b	71	21.33 ± 0.55 c	89	13.83 ± 0.36 d

Different letters within the same rows indicate significantly different temperatures determined by the paired bootstrap test with 100,000 resamplings ($P < 0.05$).

Population projections of *O. strigicollis* in response to temperature

Data from the life table study were used to project the population growth of *O. strigicollis* with an initial number of 10 viable eggs (Figure 6). The population increased faster at 27°C than at 18.5, 23.5, and 33°C. After 40 days, the total numbers of *O. strigicollis* produced under 18.5, 23.5, 27, and 33°C conditions were 8 (0 eggs, 6 nymphs, 1 female adult, and 1 male adult), 181 (21 eggs, 152 nymphs, 4 female adults and 4 male adults), 3,308 (2,014 eggs, 1,115 nymphs, 109 female adults, and 70 male adults), and 855 (430 eggs, 299 nymphs, 73 female adults, and 53 male adults), respectively.

With an initial population of five pairs of newly hatched adults, after 40 days, the total numbers of *O. strigicollis* produced at 18.5, 23.5, 27, and 33°C were 223 (79 eggs, 137 nymphs, 4 female adults, and 3 male adults), 2,926 (1,695 eggs, 1,062 nymphs, 87 female adults, and 82 male adults), 12,888 (5,380 eggs, 5,936 nymphs, 986 female adults, and 586 male adults), and 3,085 (564 eggs, 2,195 nymphs, 184 female adults, and 142 male adults), respectively (Figure 7).

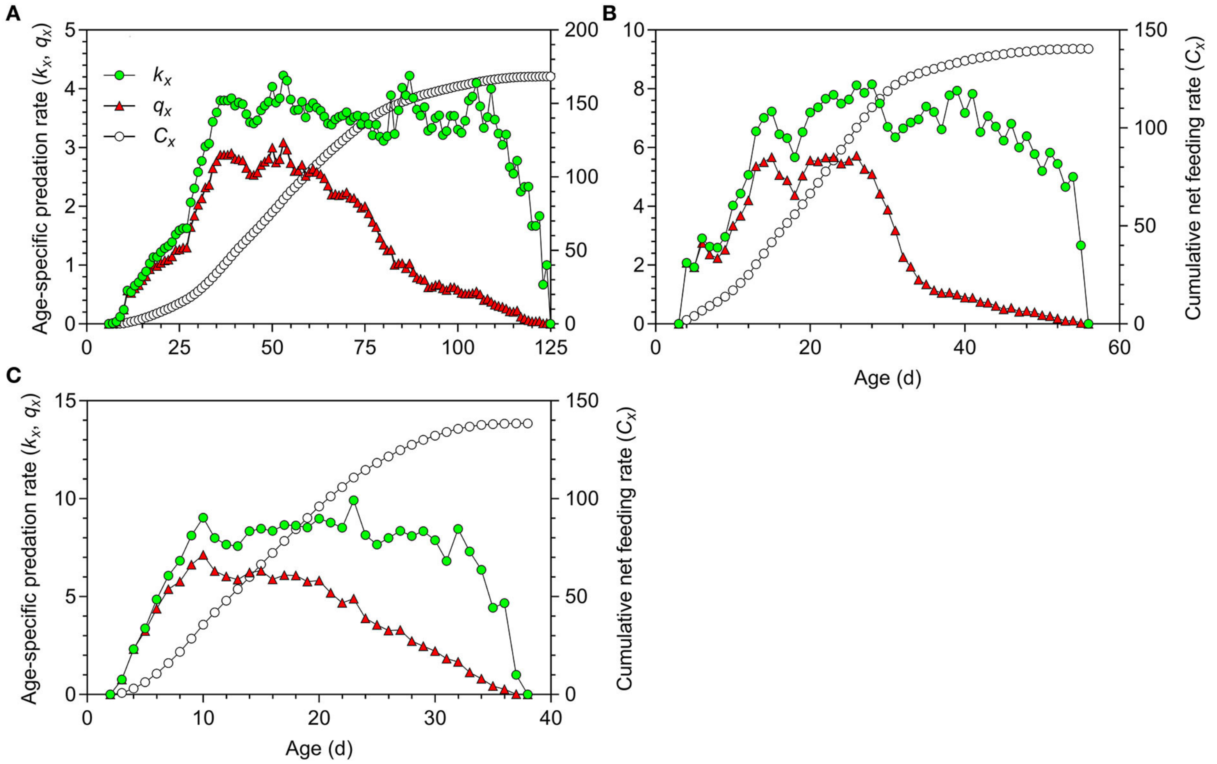

FIGURE 5
Age-specific predation rate (k_x), age-specific net predation rate (q_x) and cumulative predation rate (C_x) of *Orius strigicollis* fed on *Frankliniella occidentalis* at 18.5°C **(A)**, 23.5°C **(B)**, and 27°C **(C)**.

Discussion

Effect of temperature on the development and fecundity of *O. strigicollis*

Variability in temperature conditions can directly affect the physiology, survival, fecundity, behavior, and other characteristics of insects (Harrison et al., 2012; González-Tokman et al., 2020), ultimately affecting population dynamics (Bai et al., 2022). The development rate of insects is temperature-dependent (Bai et al., 2022), and the developmental duration of *Orius* can be significantly affected by this abiotic factor. Consistent with these findings, our results showed that temperature had a significant effect on the developmental duration of *O. strigicollis*. The prolongation of developmental duration observed at decreased temperatures may be due to a decrease in insect metabolism at lower temperatures (Howe, 1967; Brown et al., 2004; Garcia et al., 2007; Williams III and Roane, 2007; González-Tokman et al., 2020). Thus, the adult preoviposition period (APOP), total preoviposition period (TPOP), oviposition days (O_d), and mean generation time (T) exhibited temperature dependence (Table 2).

Low temperatures resulted in fewer eggs laid per day by *O. strigicollis*, similar to *Bicyclus anynana*, which laid fewer eggs per day at lower temperatures than at higher temperatures (Geister et al., 2008). However, the mean number of eggs laid per *O. strigicollis* female over a lifetime revealed no significant difference between 18.5, 23.5, and 27°C when considering the longevity and survival of adults. The fecundity of insects is limited under high-temperature conditions (Harrison et al., 2012). For example, when the temperature was 32–36°C, the fecundity of *Orius tantillus* decreased substantially (Ballal et al., 2017), and the fecundity of both *Orius laevigatus* and *Orius albidipennis* decreased substantially as the temperature was increased to 35°C (Cocuzza et al., 1997; Sanchez and Lacasa, 2002). As a result of physiological trade-offs, insects may need to reduce their fecundity to ensure high-quality egg production (Berger et al., 2008). Fand et al. (2015) also reported reduced adult longevity at high temperatures, as well as a shortening of the reproductive phase with decreased oviposition. In the current study, the high temperature had an obvious effect on the fecundity of *O. strigicollis*, resulting in a significantly low net reproductive rate (R_0 = 6.02 offspring/individual at 33°C).

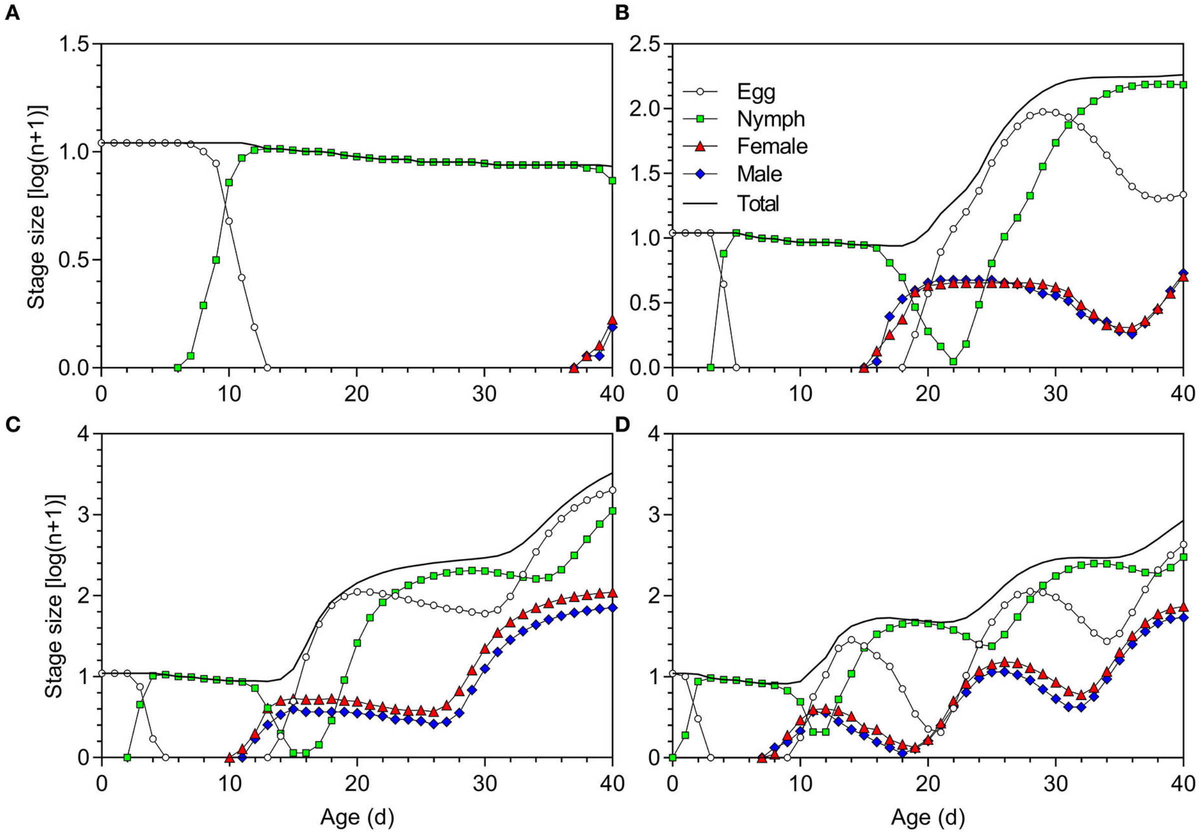

FIGURE 6
Population projection of *Orius strigicollis* fed on *Frankliniella occidentalis* at **(A)** 18.5°C, **(B)** 23.5°C, **(C)** 27°C, and **(D)** 33°C starting with an initial population of 10 viable eggs.

Effect of temperature on the population parameters of *O. strigicollis*

Previous studies by Tuan et al. (2016) and Ding et al. (2021) showed that the intrinsic rate of increase (r) and finite rate of increase (λ) of *O. strigicollis* fed on *Cadra cautella* ($r = 0.1677$ day^{-1}, $\lambda = 1.1826$ day^{-1}) and *Frankliniella intonsa* nymphs ($r = 0.1437$ day^{-1}, $\lambda = 1.1546$ day^{-1}) at 25°C were similar, and these rates were also similar to those of *O. strigicollis* fed on WFT nymphs at 27°C in this study ($r = 0.1682$ day^{-1}, $\lambda = 1.1833$ day^{-1}). The temperature had obvious effects on developmental duration, longevity, survival, and fecundity; thus, both the population parameters of r and λ were also temperature dependent (Yu J. K., et al., 2013; Ali et al., 2020). For *O. strigicollis* reared at 18.5°C and 23.5°C, a slow developmental rate (Supplementary Table 1) was the primary factor resulting in the low rate of population increase. However, despite the low fecundity of *O. strigicollis* at 33°C, the short preadult duration and adult longevity enhanced population growth, resulting in large increases in these rates ($r = 0.1298$ day^{-1}, $\lambda = 1.1387$ day^{-1}). These findings are similar to the results of a previous study, where the recorded values were 0.12 day^{-1} (r) and 1.13 day^{-1} (λ) for *O. strigicollis* reared on eggs of *Pectinophora gossypiella* at 31°C (Ali et al., 2020).

Effects of temperature on the predation rates of *O. strigicollis*

Temperature strongly affects the predation ability of predaceous insects, as frequently confirmed by the functional response (Sørensen et al., 2013; Ge et al., 2018; Rehman et al., 2020; Bai et al., 2022). As the temperature increases within a tolerable temperature range, physiological metabolism increases, and predators consume more prey to meet these energetic demands (Schwarz and Frank, 2019; González-Tokman et al., 2020). Similarly, the mean predation rates per day (D_j) of both nymphs and adults of *O. strigicollis* on WFT increased as the temperature increased (Table 4). The finite predation rate (ω) can be used to assess the potential predation of natural enemies (Chi et al., 2011; Yu J. K., et al., 2013); this rate showed an increase with increasing temperature in the

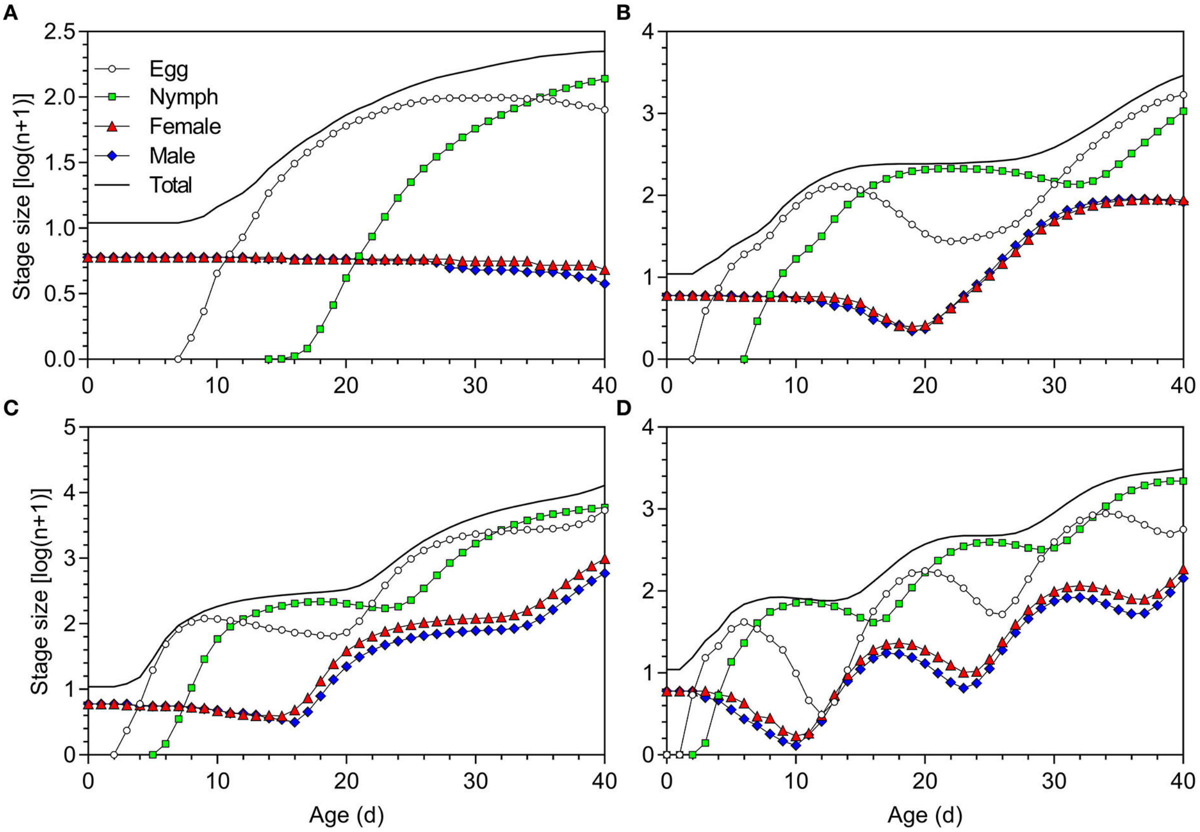

FIGURE 7
Population projection of *Orius strigicollis* fed on *Frankliniella occidentalis* at **(A)** 18.5°C, **(B)** 23.5°C, **(C)** 27°C, and **(D)** 33°C starting with an initial population of five paired adults.

current study, similar to trends in previous studies (Sørensen et al., 2013; Helgadóttir et al., 2017; Ge et al., 2018). When considering the survival rate, longevity, and predation rate, higher net predation rates (C_0) were observed at 18.5 and 23.5°C than at 27°C, similar to the result reported by Yu J. K., et al. (2013). Additionally, when developing at low temperatures, some insects can physiologically adapt by accumulating energy reserves to improve cold stress tolerance (Denlinger and Lee, 2010). Thus, *O. strigicollis* reared at 18.5°C may need to accumulate more energy reserves for survival by increasing the predation rate. Accordingly, the transformation rate (Q_p) was highest at the lowest temperature (18.5°C), allowing individuals to maximize fitness at this temperature.

Population projections and release

The computer simulation in this study showed the stage structure and population dynamics of *O. strigicollis* in response to different temperatures, providing a reference for the field application of this natural enemy under various environmental temperature conditions. The optimal predator stage, release time, and release ratio of natural enemies at various temperatures can be determined based on population projections (Yu L. Y., et al., 2013; Mou et al., 2015; Ding et al., 2021) to ensure the establishment of sufficient natural enemy populations for pest population suppression (Janssen and Sabelis, 2015; Mendoza et al., 2021). Obviously, the developmental stage of natural enemies released can affect population dynamics, and the *O. strigicollis* population increased faster when initiated using five pairs of adults than when using 10 viable eggs, similar to the results reported by Ding et al. (2021). As the predation rate was also age-dependent (Table 4), the release of adults or nymphs of *O. strigicollis* with a high predation rate is favorable for achieving immediate pest control (van Lenteren et al., 2018; Ding et al., 2021). In addition, the preventative release of natural enemies (one aspect of ABC) by introducing natural enemies in the greenhouse first and supporting natural enemy establishment before pest arrival has also been proposed (Messelink et al., 2014; van Lenteren et al., 2018; Pijnakker et al., 2020). This method facilitates the establishment of an adequate population of natural enemies,

TABLE 4 Predation rate for daily (D_j) of *Orius strigicollis* fed on *Frankliniella occidentalis* at different temperatures.

Development stage	18.5°C		23.5°C		27°C	
	n	Mean ± (SE)	*N*	Mean ± (SE)	*n*	Mean ± (SE)
N1	74	0.63 ± 0.02 c	88	2.09 ± 0.06 b	71	2.34 ± 0.09 a
N2	66	1.19 ± 0.03 c	80	2.52 ± 0.06 b	65	4.40 ± 0.17 a
N3	61	1.50 ± 0.04 c	73	3.79 ±0.09 b	60	6.64 ± 0.03 a
N4	58	2.58 ±0.08 c	73	5.85 ± 0.18 b	58	8.37± 0.27 a
N5	56	3.71 ±0.08 c	66	6.92 ± 0.14 b	56	8.09 ± 0.19 a
Preadult	74	1.46 ± 0.04 c	88	3.21 ± 0.07 b	71	4.11 ± 0.12 a
All adult	55	3.60 ± 0.13 c	66	7.28 ± 0.32 b	54	8.34 ± 0.33 a
Female adult	26	4.40 ± 0.11 b	32	9.44 ± 0.19 a	33	9.88 ± 0.19 a
Male adult	29	2.72 ± 0.09 c	34	4.91 ± 0.19 b	21	5.75 ± 0.23 a

Different letters within the same rows indicate significantly different temperatures determined by the paired bootstrap test with 100,000 resamplings ($P < 0.05$).

TABLE 5 Predation rate for the stage of *Orius strigicollis* survived (P_j) fed on *Frankliniella occidentalis* at different temperatures.

Development stage	18.5°C		23.5°C		27°C	
	n	Mean ± (SE)	*N*	Mean ± (SE)	*n*	Mean ± (SE)
N1	66	4.18 ± 0.15 a	80	4.45 ± 0.10 a	65	4.45 ± 0.18 a
N2	60	6.90 ± 0.19 b	73	8.08 ± 0.27 a	60	7.35 ± 0.26 ab
N3	58	8.16 ± 0.29 c	73	11.33 ±0.35 a	58	9.55 ± 0.38 b
N4	57	14.93 ±0.68 a	69	13.09 ± 0.42 b	56	12.63 ± 0.49 b
N5	55	35.36 ±0.84 a	66	26.62 ± 0.72 b	54	26.20 ± 0.71 b
Preadult	55	69.96 ± 0.98 a	66	63.61 ± 1.01 b	54	60.28 ± 1.17 c
All adult	55	152.96 ± 0.12 a	66	117.76 ± 9.00 b	54	116.90 ± 8.68 b
Female adult	26	198.87 ±13.21 a	32	164.92 ± 12.77 a	33	141.93 ± 11.28 b
Male adult	29	108.69 ±11.71 a	34	73.38 ± 6.74 b	21	77.55 ± 8.33 b

Different letters within the same rows indicate significantly different temperatures determined by the paired bootstrap test with 100,000 resamplings ($P < 0.05$).

TABLE 6 Parameters of *Orius strigicollis* preying (Mean ± SE) on *Frankliniella occidentalis* at different temperatures.

Parameter	18.5°C	23.5°C	27°C
Net predation rate, C_0 (prey/predator)	168.39 ± 13.36 a	140.49 ± 10.21 a	138.39 ± 10.72 b
Finite predation rate, ω (d^{-1})	1.14 ± 0.04 c	2.63 ± 0.09 b	3.24 ± 0.14 a
Transformation rate, Q_p	8.22 ± 1.42 a	6.03 ± 0.98 a	3.81 ± 0.58 a

Different letters within the same rows indicate significantly different temperatures determined by the paired bootstrap test with 100,000 resamplings ($P < 0.05$).

allowing predator synchronization with pest population growth. Thus, in the preventative release of this natural enemy, both the effects of environmental temperatures and the release stage of *O. strigicollis* on the population dynamics should be considered to determine the timing of release to prevent thrips outbreaks.

Integrated analysis of the demography and predation rate of the natural enemy in response to temperature can facilitate the biocontrol efficacy of natural enemy release for pest control. This study comprehensively described the demographic characteristics and predation rate of *O. strigicollis* in response to temperature. Increasing temperature promoted the development and shortened the longevity of *O. strigicollis*. At 27°C and 33°C, *O. strigicollis* exhibited rapid population growth; in contrast, relatively low temperatures, especially 18.5°C, led to slow population growth. The predation rates of *O. strigicollis* in various developmental stages varied greatly at different temperatures. Consequently, when releasing *O. strigicollis* in the field to control WFT, both environmental temperature and developmental stage should be taken into consideration to establish sufficient populations.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. Requests to access these datasets should be directed to luybcn@163.com.

Author contributions

XR, LZ, and YL conceived the research, designed experiments, and wrote the manuscript. XL, JH, and ZZ collected and prepared material. XR, JZ, LC, and SZ performed experiments and collected data. XR, XL, and MH analyzed data. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2022.1026115/full#supplementary-material

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EDITED BY
Wen Xie,
Insititute of Vegetables and Flowers (CAAS), China

REVIEWED BY
Xingyuan Men,
Shandong Academy of Agricultural Sciences, China
Shiliang Liu,
Beijing Normal University, China
Paul Glaum,
Waterborne Environmental, Inc., United States

*CORRESPONDENCE
Ruonan Li
✉ rnli@rcees.ac.cn

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Landscape composition and configuration relatively affect invasive pest and its associator across multiple spatial scales

Zhi Wen[1], Quanfeng Yang[1], Binbin Huang[1], Lu Zhang[1,2], Hua Zheng[1,2], Yusheng Shen[2,3,4], Yanzheng Yang[1,2], Zhiyun Ouyang[1,2] and Ruonan Li[1,2]*

[1]State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China, [2]College of Resource and Environment, University of Chinese Academy of Sciences, Beijing, China, [3]Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China, [4]Xiamen Key Lab of Urban Metabolism, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China

Landscape structures affect pests, depending on compositional heterogeneity (the number and proportions of different habitats), configurational heterogeneity (spatial arrangement of habitats), and spatial scales. However, there is limited information on the relative effects of compositional and configurational heterogeneity on invasive pests and their associates (species that can benefit from invasive pests), and how they vary across spatial scales. In this study, we assayed the invasive pest *Bactrocera dorsalis* (Hendel) and its associated fly *Drosophila melanogaster* in 15 landscapes centered on mango orchards. We calculated landscape composition (forest percentage, mango percentage, and Shannon's diversity) and configuration (edge density) using two methods: spatial distance scales and combined scales. Spatial distance scales included buffer rings with radii of 0.5, 1.0, and 1.5 km, and combined scales referred to cutting or not cutting a smaller ring from larger ones. Our results shown that compositional heterogeneity positively affected *B. dorsalis* and *D. melanogaster* due to forest cover percentage, whereas configurational heterogeneity with high edge density negative effect on *B. dorsalis*. Forest cover had less of an effect on *B. dorsalis* than configurational heterogeneity, but the opposite effect was observed for *D. melanogaster*. Importantly, the direction and strength of forest cover and configurational heterogeneity to species did not vary with spatial distance scales or spatial combined scales. Thus, compositional and configurational heterogeneity exhibit differential effects on this invasive pest and its associator, and revealed that the relative effects of landscape structures are consistent across multiple scales. These results provide new insights into landscape effects on interconnected species using a diverse spatial-scale approach.

KEYWORDS

landscape complexity, landscape fragmentation, landscape effects, invasive species, pest control, multiscale method

1. Introduction

Land use change and intensification have led to habitat destruction and fragmented landscapes, potentially exacerbating biodiversity loss and pest outbreaks (Sirami et al., 2019; Tougeron et al., 2022). Landscape heterogeneity, constituted by the types of habitats surrounding agricultural lands and their spatial arrangement, has been recognized as important for pest control (Clemente-Orta et al., 2020; Paredes et al., 2021). However, previous studies have focused on the impact of landscape structures on native pests while paying little attention to invasive pests and their effects on interconnected insects (e.g., commensalism) (Schmidt et al., 2019; Shi et al., 2021). Understanding of how landscape heterogeneity regulates pests and their associates (species with interactions with pests) remains a core topic in revealing landscape effects (Gagic et al., 2021; Shi et al., 2021) and an urgent need for developing conservation strategies (Rios et al., 2021; Ratto et al., 2022).

Landscape effects on pests depend on compositional heterogeneity and configurational heterogeneity (Fahrig et al., 2011; Kheirodin et al., 2020; Ouyang et al., 2020; Zhang et al., 2021), but there is no consensus on their relative contributions for pest control (Rybicki et al., 2020; Metzger et al., 2021; Saura, 2021), perhaps the lack of consensus is due to the functional trait and different needs of the species studied (Aristizábal and Metzger, 2018; Martin et al., 2019). The habitat amount hypothesis states that the habitat amount in the landscape surrounding a sample site determines species abundance (Fahrig, 2013; Rios et al., 2021; Malagnini et al., 2022). Several studies support this hypothesis; for example, coffee coverage in agricultural landscapes is positively correlated with coffee berry borer (Aristizábal and Metzger, 2018), as more resources are available to the pest. In addition, the island biogeography theory argues that landscape configurations with high habitat fragmentation are critical to species (Rybicki et al., 2020; Saura, 2021). Generally, habitat fragmentation is detrimental to individual species abundance (Heidrich et al., 2020; Rybicki et al., 2020), as it forms small isolated islands that support smaller communities, increasing the probability of inbreeding and extinction (MacDonald et al., 2018). The negative correlation between habitat fragmentation (e.g., edge density) and pests observed in previous studies supports this theory (Bosem Baillod et al., 2017); however, contradictory results have shown that habitat fragmentation is positively related to species abundance (Fahrig, 2017; Fletcher et al., 2018), which is attributable to, but not limited to, habitat type diversity and spatial scale of effect (Aristizábal and Metzger, 2018; Fletcher et al., 2018; MacDonald et al., 2018).

Differences in spatial scale may significantly affect the impact of habitat cover and fragmentation on pests (Aristizábal and Metzger, 2018; Da Silva Carneiro et al., 2022). Landscape structures may even have opposite effects on pests at different spatial distance scales (the radius of the distance from the sampling site forms different spatial buffer rings, Figure 1B), as the dispersal ability of pests is limited due to their functional traits. For example, the correlation between forest cover and coffee berry borers changes from positive to negative as the spatial distance changes from 300 m to 2 km (Aristizábal and Metzger, 2018). In addition to the spatial distance scale, the spatial combined scale formed by the small distance buffer rings (e.g., 0.5 km radius) nested within the large distance buffer rings (e.g., 1.5 km radius, Figure 1B) may affect the species distribution (Da Silva Carneiro et al., 2022). Delineating the scope of the landscape by spatial distance is insufficient to elucidate the landscape effects, because the surrounding landscape effects is not clarified by removing small nested circles. Moreover, the expansion of spatial distance potentially increases new habitat types and spatial arrangements (Da Silva Carneiro et al., 2022), and it is difficult to elucidate the landscape effects of these new elements without decoupling spatial scales (removing the small buffer ring from the large one). Unfortunately, almost all previous studies have focused on the spatial distance scale while ignoring the spatial decoupled of effect (Aristizábal and Metzger, 2018; Kheirodin et al., 2020), which limits the understanding of how landscape affects pests.

In this study, we explored the relative effects of landscape composition and configuration on pests at multiple spatial scales. We targeted a pest fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), in mango (*Mangifera indica* Linn) orchards. Mangoes are a popular fruit worldwide, and *B. dorsalis* is a serious threat to mango yield and quality (Grechi et al., 2022). In African countries invaded by *B. dorsalis*, the proportion of damaged mango fruits that have been recorded can be as high as 78%, causing serious economic losses to local small farmers (Cugala et al., 2020). As an invasive pest in China and other regions (Liu et al., 2019; Grechi et al., 2022), *B. dorsalis* females pierce the skin of fruit during oviposition. Larvae that develop inside the fruit tend to feed on the most nutritious part of the pulp, which leads to fruit damage. Larvae drop from the host and burrow into soil to form pupae. Adults feed mostly on nectar, but some also draw nutrients from pollen and rotting fruit (Liu et al., 2019). Owing to its strong ability to survive and reproduce, *B. dorsalis* inevitably affects native species. As another fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae) can benefit from *B. dorsalis* because *D. melanogaster* can obtain food and reproduce from mango fruits infested by *B. dorsalis.* Therefore, *D. melanogaster* is defined as an associator of *B. dorsalis.*

We selected 15 landscapes with mango orchards at their center on Hainan Island (Figure 1), the main mango fruit-producing area in China, which has been invaded by *B. dorsalis* (Liu et al., 2019). We measured *B. dorsalis* and *D. melanogaster* abundances in each mango orchard and its surrounding habitats. The compositional and configurational heterogeneity in each landscape were characterized on a spatial distance scale and spatial combined (coupled and decoupled) scales. We hypothesized that the impact of landscape heterogeneity on an invasive pest and its associator varies with spatial scale and examined the following questions: (1) How do compositional and configurational heterogeneity differ in their impact on the invasive pest and its associator? (2) Do landscape variables in relation to the invasive pest and its associator vary with spatial distance scales, and (3) spatial combined scales?

2. Materials and methods

2.1. Study site and landscape settings

This study was conducted in the southwestern region of Hainan Island, China (18°45′ N, 109°17′ E). The area has a tropical marine monsoon climate. The average annual temperature is 19–26°C. The mean annual precipitation is 1,400–1,800 mm. Land-use patches are dominated by smallholders, forming a highly heterogeneous mosaic landscape mainly comprised of forest, rubber (*Hevea brasiliensis*), mango, longan (*Dimocarpus longan*), papaya (*Chaenomeles sinensis*), areca (*Areca catechu* L.), and farmland (rice and vegetables) patches. Mango orchards, which are managed similarly and are the main income source for many smallholders in the region, have been affected by the invasive pest *B. dorsalis* for many years. *B. dorsalis* is thought to have invaded Hainan Island as early as 1934 (Liu et al., 2019). Smallholders would use insecticides to exterminate *B. dorsalis* 1–2 times a year during the near-ripening period of mangoes.

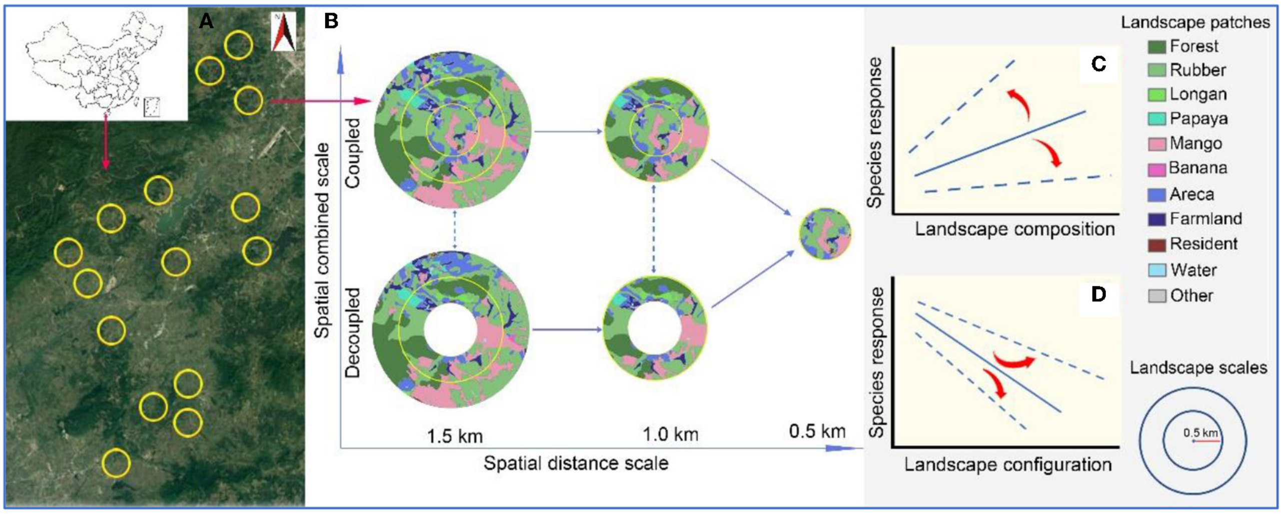

FIGURE 1
Schematic diagram of the study area and landscape settings. The yellow circles (1.5 km radius) in the left panel **(A)** indicate the spatial distribution of the 15 landscapes. The right panel **(B)** shows the processing of the landscape at spatial distance and combined scale. Distance scales refer to buffer rings with 0.5, 1, and 1.5 km radius around the central mango patch, respectively [x-coordinate, **(B)**], and the combined scale refers to whether to remove the ring with 0.5 km [y-coordinate, **(B)**]. **(C, D)** Indicate the potential impact of spatial scale on landscape heterogeneity-species relationships.

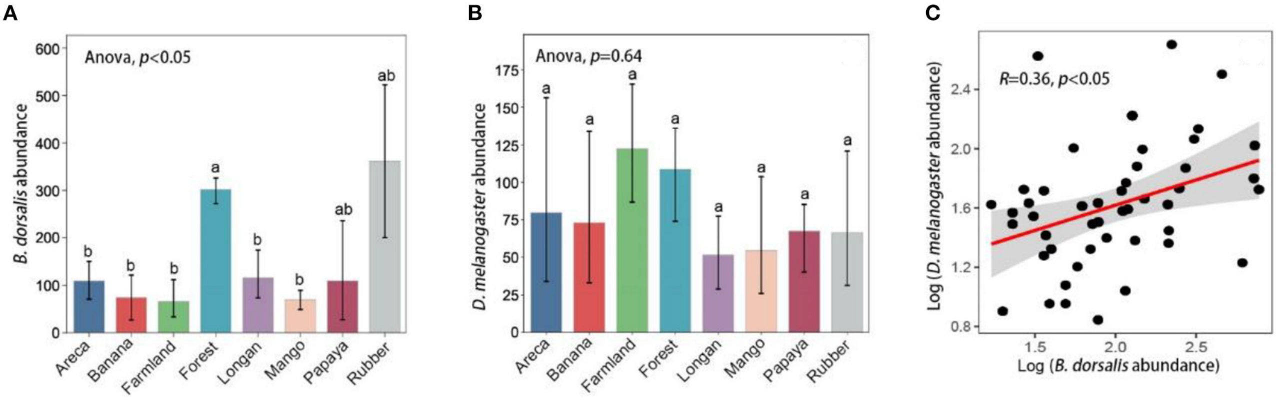

FIGURE 2
Species abundance differences among habitats. **(A)** Indicates *Bactrocera dorsalis* abundance, **(B)** indicates *Drosophila melanogaster* abundance, and different lowercase letters on the histogram indicate significant differences. **(C)** Indicates the correlation between *B. dorsalis* and *D. melanogaster* abundance by correlation analysis.

Taking the mango patch as the center, we set 15 landscapes as a gradient based on forest cover, with a radius of 1.5 km surrounding the mangoes (Figure 1A). This landscape radius was chosen based on previous studies that observed traces of multiple pest activity (Gardiner et al., 2009; González et al., 2020). Among the selected landscapes, forest cover percentage varied from 0 to 30%, with varying degrees of landscape fragmentation.

2.2. Compositional and configurational heterogeneity

First, we obtained a high-resolution geographic image map for each landscape based on their coordinates using ArcGIS 10.2 software. We divided the landscape patches into 11 categories [forest, mango, rubber, longan, areca, banana, papaya, farmland, water area, residential land, and others (Figure 1B)] and then used ArcGIS 10.2 software to outline each patch. The patches were identified by combining high-resolution satellite images (0.83 m) and data from field surveys conducted in June–August 2021, after which the vector graphics were converted into raster maps to calculate compositional and configurational heterogeneity metrics. We used FRAGSTATS 4.2 software (McGarigal et al., 2012) to calculate the landscape indices.

Since longan, papaya, farmland and banana have a small percentage of area in the landscape, while forest, mango, rubber and areca are the main land types that make up the composition of the landscape, the compositional heterogeneity indicators included mango cover percentage (%), forest%, rubber%, areca%, and Shannon's diversity index. Configurational heterogeneity was indicated by edge density, which can reflect the degree of landscape fragmentation (Martin et al., 2019).

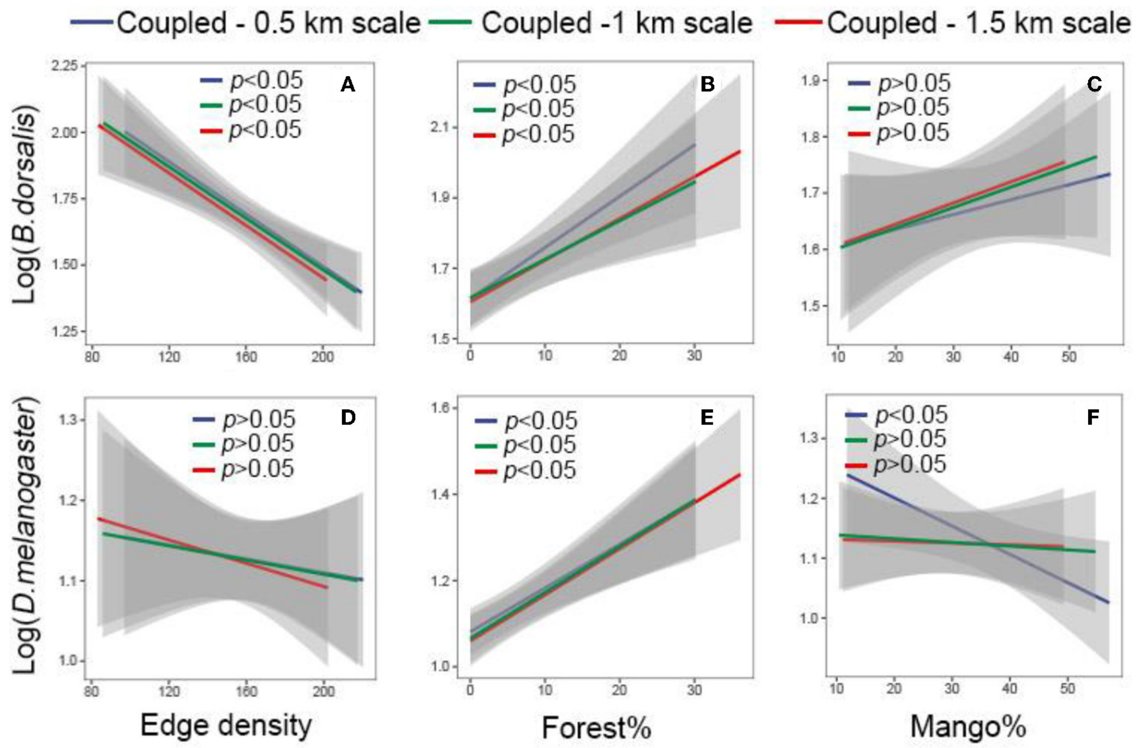

FIGURE 3
Linear relationships between landscape heterogeneity and species at spatial distance scales (0.5, 1, and 1.5 km). **(A–C)** Indicate log-transformed *Bactrocera dorsalis* abundance; **(D–F)** indicated log-transformed *Drosophila melanogaster* abundance. The blue, green, and red lines represent the spatial distance scales of 0.5, 1, and 1.5 km, respectively. The coupled results are shown, and the decoupled results can be known from Supplementary Figure 2.

2.3. Landscape spatial scales

Two spatial scales were set: spatial distance scale and spatial combined scale. The spatial distance scale refers to the circle of buffer rings centered on the mango patch with radii of 0.5, 1, and 1.5 km, respectively (Figure 1B), which can clarify the effect of landscape variables with spatial distance.

The spatial combined scales were defined based on whether the buffer ring with a radius of 0.5 km was cut off from the larger 1 and 1.5 km rings. The nested landscape formed without being cut off was called the coupled (Figure 1B), and the central blank landscape formed by cutting off was called a decoupled. Thus, the spatial combined scale contains two categories of coupled and decoupled (Figure 1B). Combined scales were used to explore the effects of surrounding habitat composition and configuration at different distances.

2.4. Species sampling

In September 2021, the newly grown leaves of mango trees in the current year were in the mature stage. The invasive pest (*B. dorsalis*) and its associator (*D. melanogaster*) were identified in mango patches at the center of each landscape and other surrounding patches (such as forest and rubber) using yellow sticky traps (length × width: 20 × 25 cm) and yellow funnel traps (attractant: methyl eugenol). Three yellow sticky traps and three funnel traps were set at a height of ∼1–2 m above the ground and were displayed at least 3 m apart within each patch. All traps were placed at least 6 m from the edge of each patch to reduce edge effects. Except for 15 mango patches at the center of the landscape, other surrounding patches were randomly selected in each landscape to determine whether the land cover types were habitats for *B. dorsalis* and *D. melanogaster*. In total, 75 patches were investigated in 15 landscapes, including 4 forest, 15 mango, 15 areca, 13 rubber, 9 farmland, 9 longan, 7 banana, and 3 papaya patches; thus, we placed 225 yellow sticky traps, and funnel traps, respectively. After 3 days, we collected all yellow sticky and funnel traps and counted the number of each species.

During the flowering (October 2021), young fruit (November 2021), and near-ripe stages (March 2022) of mangoes, we measured the invasive pest and its associator in central mango patches using two yellow traps methods by the same sampling method as in September 2021. Therefore, the numbers of each species in mango patches were determined four times. The species abundance of each patch measured each time was obtained by summing the two methods, following previous study (Perrot et al., 2022).

2.5. Statistical analysis

At the patch level, we confirmed the presence of both species in the selected habitat types, and then analyzed the differences in *B. dorsalis* and *D. melanogaster* abundances among habitats using ANOVA. Multiple comparisons were then made using the Tamhane's T2 due to the heterogeneity of variance in the species abundance data. We explored the correlation between *B. dorsalis* and *D. melanogaster* abundance using linear regression analysis across all patches. Species abundance was log-transformed before doing the regression analysis, as were the following regressions and correlation analysis.

At the landscape level, we first removed the covariance between landscape variables in two steps. (a) Pearson's correlation analysis was performed on the landscape variables at each spatial scale (Supplementary Figure 1), and only those variables with weak correlations ($r < 0.65$) were retained (Da Silva Carneiro et al., 2022); (b) The retained landscape variables were subjected to multiple regression analysis to log-transformed species abundance, and the landscape variables were further filtered by VIF<2 (Perrot et al., 2022). The landscape variables that were thus screened were edge density, forest cover percentage (%), mango%, and Shannon's diversity (Supplementary Table 1). The general linear model was then used to analyze the relationship between each landscape variable and species abundance, with landscape variables as fixed effects, species abundance as response variables, and sampling site and month as covariates in the spatial distance and combined scales.

To explore the effects of spatial distance and combined scales on the landscape variable-species abundance relationship, we explored differences in linearly fitted relationships between landscape variables (Supplementary Table 1) and species abundance using grouped regression. The spatial distance scale varies from 0.5 to 1 km and then to 1.5 km. Spatial combined scales for coupled and decoupled were compared at distance scales of 1 and 1.5 km, respectively. Differences in linearly fitted relationships were checked using the Chow test (Zeileis et al., 2002; Wen et al., 2022).

To reveal the optimal landscape variables and spatial scales, we subjected all landscape variables (Supplementary Table 1) and species (*B. dorsalis* and *D. melanogaster*) abundance to multiple linear regression analysis in a stepwise approach, with landscape variables as independent variables and species abundance as dependent variables. Standardized regression coefficients were used to compare the relative effects of landscape variables on species. All of the above data analyses were conducted using IBM SPSS software (version 21.0).

3. Results

A total of 40 594 *Bactrocera dorsalis* and 10 438 *Drosophila melanogaster* specimens were collected during the sampling period. Both species were captured in the selected habitat types (Figure 2A). *B. dorsalis* abundance in forests was significantly higher than that in mango orchards ($P < 0.05$, Figure 2A), but there was no significant difference between mangoes and other habitats ($P < 0.05$, Figure 2A). There were no significant differences in *D. melanogaster* abundance among the habitats ($P > 0.05$, Figure 2B). *B. dorsalis* and *D. melanogaster* abundances showed a significant positive correlation across the landscape patches ($P < 0.05$, Figure 2C).

3.1. Impact of landscape heterogeneity on pests

Configurational heterogeneity (edge density) was negatively correlated with *B. dorsalis* abundance ($P < 0.05$, Figure 3A, Supplementary Figure 2A), but the correlation with *D. melanogaster* was insignificant ($P > 0.05$, Figure 3D, Supplementary Figure 2D). In contrast, compositional heterogeneity was positively correlated with *B. dorsalis* and *D. melanogaster* abundance owing to an increase in the percentage of forest cover ($P < 0.05$, Figures 3B, E, Supplementary Figures 2B, E). The percentage of mango cover was not significantly associated with either species at 1 and 1.5 km scales ($P > 0.05$, Figures 3C, F).

3.2. Spatial distance scale effects of landscape heterogeneity on pests

Negative relationships between edge density with *B. dorsalis* abundance was observed across spatial distance scales of 0.5, 1, and 1.5 km (Figure 3A, Figure 2A). In addition, there were no significant changes in the slopes of these fitted lines (Figure 3A), implying that variation in the strength of these indicator effects is limited. Positive correlation between forest cover percentage with *B. dorsalis* and *D. melanogaster* abundances did not change with spatial distance scale from 0.5, 1 to 1.5 km (Figures 3B, E). The slope of the fitted lines between forest cover and species abundance did not change significantly at the scales of 0.5,1, and 1.5 km (Figures 3B, E), indicating that the effect of forest cover had limited variation in intensity. Overall, spatial distance scales did not alter the direction and intensity of compositional and configurational heterogeneity affecting the species (Figure 3).

3.3. Spatial combined scale effects of landscape heterogeneity on pests

At spatial coupled and decoupled, the direction of linear relationships between edge density with *B. dorsalis* abundance was consistent, regardless of 1 or 1.5 km space distances (Figure 3). In addition, there was no difference in the strength of the linear relationship, as the differences in the regression coefficients of the fitted straight lines were insignificant ($P > 0.05$, Table 1, Supplementary Table 2). Similarly, the direction and strength of the correlation between forest percentage with *B. dorsalis* and *D. melanogaster* abundance did not change from spatial coupled (Figures 3B, E) to decoupled, regardless of 1 km (Table 1) or 1.5 km space distances (Supplementary Table 2). Therefore, spatially combined scales had limited effects on compositional and configurational heterogeneity effects.

Considering all landscape variables in spatial distance and combined scales, edge density at coupled 1 km scale and forest cover at coupled 0.5 km scale were important factors for *B. dorsalis*, and the importance of the former (absolute value of the standardized coefficient) was higher than the latter (Table 2). For *D. melanogaster*, the proportion of forest cover at coupled 1 km scale was more important than edge density at coupled 1 km scale, although the latter had a significant effect (Table 2).

4. Discussion

This study aimed to explore at multiple spatial scales how landscape heterogeneity affects an invasive pest (*B. dorsalis*) and an associated fly insect (*D. melanogaster*, hereafter "associator") that could benefit from the invasive pest. The results showed that the

TABLE 1 Grouped regression results show differences in the fitting linear relationship between landscape heterogeneity with pests from spatial coupled to decoupled.

Species abundance	Landscape heterogeneity	Coupled	Decoupled	Δ*b* (*b1*-*b2*)	*t*	*p*
		b1	*b2*			
B. dorsalis	Edge density	−0.005	−0.005	−0.000	−0.070	0.944
	Forest%	0.012	0.010	0.002	0.219	0.827
D. melanogaster	Forest%	0.014	0.013	0.001	0.195	0.846

b1 and b2 refer to the linear regression coefficients of landscape structures on species at landscape coupled and decoupled on a 1 km distance scale, respectively. Differences between coupled with decoupled were checked using the Chow test.

effects of landscape heterogeneity on the invasive pest were opposite in compositional and configurational heterogeneity, with the former having positive and the latter having negative effects. Compared with compositional heterogeneity, configurational heterogeneity had higher and lower relative contributions to the invasive pest and associator, respectively, but did not vary with diverse spatial scales. Thus, landscape effects are species-dependent, but not spatial scale-dependent.

4.1. Effects of compositional and configurational heterogeneity

Compositional heterogeneity was positively correlated with the invasive pest and associator dependent on forest cover rather than other habitats, which differs from the results of previous studies showing that forest cover is often thought to reduce pest abundance (Medeiros et al., 2019; González et al., 2020). For example, forest cover is negatively associated with a coffee leaf pest (*Leucoptera coffeella*) (Medeiros et al., 2019) and fruit pest (coffee berry borer) (Aristizábal and Metzger, 2018). This conflicting result may be attributed to the differences in the ability of forests to control native and invasive pests. For native pests, various factors exist in the forests that limit their populations, such as competitors and natural predators (Henri et al., 2015; Aristizábal and Metzger, 2018). For invasive pests, however, there is not only a lack of competitors and natural enemies, but also sufficient alternative food sources, overwintering sites, and refuges provided by forests with high plant diversity (Tscharntke et al., 2016; Gurr et al., 2017; Tamburini et al., 2020). This is potentially conducive to the survival and reproduction of invasive pests, thereby increasing the number of invasive pests in agricultural land adjacent to forests. Similarly, in addition to obtaining resources from surrounding forests, the associator can better maintain populations by following the invasive pest, as shown by the significant positive correlation between the abundance of the invasive pest and the associator.

In contrast, configurational heterogeneity was negatively correlated with invasive pest abundance. Both negative and positive effects of landscape configuration on insects have been reported in previous studies (Fahrig, 2017; Martin et al., 2019). For pests, configurational heterogeneity with high edge density may reduce crop cover available as a food resource, echoing the resource concentration hypothesis (Tscharntke et al., 2016), while high edge density resulting from fragmentation promoted pest transfer from crop patches to surrounding habitat due to dispersal effects (Martin et al., 2019; Haan et al., 2020). Fragmentation increases new habitats that are more suitable for pests to survive, as in the present study, and rubber plantations are more suitable for *B. dorsalis* than mango orchards, accelerating the outward spread of *B. dorsalis*, thereby making configurational heterogeneity negatively related to this invasive pest. However, the associator was insignificantly associated with configurational heterogeneity, which may be attributed to the insignificant differences in associator abundance across habitat types (Figure 2B), that is, the number per unit area remained unchanged regardless of configurational heterogeneity. This indicates that species respond differently to landscape changes (Martin et al., 2019), even though the two species are closely related.

Notably, compositional heterogeneity contributed less to invasive pest abundance than configurational heterogeneity; however, the opposite was true for the associator. Although landscape composition with high forest cover promoted the invasive pest, invasive pest overflow from forests would be buffered by configurational heterogeneity with high edge density through dilution effects (Haan et al., 2020), similar to that observed in other insects (Martin et al., 2019; Souza et al., 2020). Other habitats (e.g., areca and banana) did not differ significantly from mango orchards in attracting invasive pests (Figure 2A), but some habitats (e.g., rubber) were more suitable for the survival of invasive pests than mango orchards. This also facilitates the escape of invasive pests in response to strong disturbances in mango orchards due to habitat fragmentation with high edge density, which provides a longer common boundary (Souza et al., 2020; Moore et al., 2022). These factors make configurational heterogeneity more important for invasive pests, supporting the habitat diversity hypothesis (MacDonald et al., 2018). In contrast, compositional heterogeneity had a greater effect on the associator, mainly because it was positively affected by forest cover, whereas edge density showed limited effects. However, at the landscape level, the relative effects of landscape variables on specific species remain elusive, as species respond to landscape heterogeneity, which varies widely across diverse landscape contexts (Martin et al., 2019; Moore et al., 2022).

4.2. Effects of spatial distance and combined scales

The results showed that the strength and direction of the relationships between compositional and configurational heterogeneity with the invasive pest and its associator did not vary with spatial distance. Some studies suggest that the effects of landscape variables on species vary with spatial distance scales (Aristizábal and Metzger, 2018; Redlich et al., 2018), which

TABLE 2 Multiple linear regression screening of landscape heterogeneity variables explaining species variation integrating spatial coupled and decoupled.

Response variable	Predictor variable	t	p	Standardized coefficient
B. dorsalis	Intercept	8.948	<0.05	–
	Edge density (coupled-1 km)	−2.312	<0.05	−0.168
	Forest% (coupled-0.5 km)	1.782	0.076	0.129
D. melanogaster	Intercept	1.953	0.052	–
	Forest% (coupled-1 km)	5.780	<0.05	0.454
	Edge density (coupled-1 km)	3.461	<0.05	0.262
	Mango% (decoupled-1 km)	2.119	<0.05	0.126

contradicts our results. This may be due to differences between species with different functional traits and dispersal abilities. Winged insects (such as *B. dorsalis* and *D. melanogaster*) can travel faster and farther than other insects because of their ability to fly (Miguet et al., 2016; Zhang et al., 2021), and generalist pests (such as *B. dorsalis*) spread farther than specialists because of extensive habitat adaptation (Miguet et al., 2016; Pan et al., 2022). In particular, invasive pests can survive in a variety of habitats and spread easily among patches owing to the formation of patch connectivity. Furthermore, the spatially coupled scales formed by small distances (e.g., 1 km) nesting within a large distance (e.g., 1.5 km) make it difficult to separate the effects from each other (Martin et al., 2016), thus making the direction and intensity of landscape variable effects constant with spatial distance.

Another novel finding of the present study is that spatially combined (coupled and decoupled) scales did not change the intensity and direction of landscape effects, which provides new insights into the effects of landscapes on pests and associators. Previous results have only shown that landscape structures control pests at spatially coupled (Aristizábal and Metzger, 2018; Kheirodin et al., 2020; Zhang et al., 2021), making it difficult to distinguish the role of surrounding landscape heterogeneity at different spatial distances from the sampled crop patches (Da Silva Carneiro et al., 2022). The comparison of results in the present study revealed that spatial coupling and decoupling did not change the landscape effects, whether the spatial distance was 1 or 1.5 km, indicating that farther and nearer distance habitat heterogeneity has similar effects on species, at least for *B. dorsalis* and *D. melanogaster*. This may be because species with a flexible diet and flight ability have an efficient dispersal capability and adaptability regardless of habitat type (Miguet et al., 2016; Zhang et al., 2021), especially for invasive pests, there may also be a lack of natural enemies in geographic space. Furthermore, habitat type and landscape variable changes were limited after spatial decoupling compared to coupling, regardless of distance.

4.3. Implications and prospects

The present findings have implications for habitat conservation and biological control, especially in the context of landscape heterogeneity due to global land-use change (Martin et al., 2019; Zheng et al., 2019). Although the results showed that forest cover was positively associated with an invasive pest, especially at a distance scale of 0.5 km radius, reducing forests to control invasive pests is not recommended. Most studies have found that forests conserve biodiversity, including plants, arthropods, birds, and mammals (Zhang et al., 2017; Aristizábal and Metzger, 2018; González et al., 2020). The benefits of controlling invasive pests by destroying forests are likely to be far less than those of biodiversity conservation by protecting forests, not to mention the fact that forests potentially provide many natural enemies to control agricultural pests (Aristizábal and Metzger, 2018; Varela et al., 2018; Martin et al., 2019). Habitat fragmentation had a negative effect on the invasive pest. Maintaining the diversity of habitat types in the surrounding landscape composition is beneficial for the control of invasive pests and can be attempted in landscape management at the 1 km landscape radius. In addition, the effects of spatial scales were not evident in the present study, it is suggested that spatial distance and combined scale should be combined to elucidate landscape effects in broader regions (Medeiros et al., 2019; Da Silva Carneiro et al., 2022), especially at spatially decoupled, as scale effects may depend on landscape contexts and species functional groups (Martin et al., 2019; Haan et al., 2020).

Pests are not only regulated by landscapes but also affected by natural enemies (Kheirodin et al., 2020; Zhang et al., 2021), which are thought to be closely related to compositional and configurational heterogeneity (Tscharntke et al., 2016; Martin et al., 2019; Kheirodin et al., 2020). Although we used a pest sampling method known to trap parasitic natural enemies (Böckmann et al., 2015), we did not capture parasitoids associated with the invasive pest that have been reported (Liu et al., 2019). It is still necessary to explore the presence or absence of other natural enemies associated with invasive pests and their responses to landscape heterogeneity at multiple spatial scales, which is important for unraveling the mechanisms of landscapes controlling invasive pests. Moreover, the effects of invasive pests on crops need to be assessed in detail, such as fruit damage rates and economic losses to smallholders, which are directly related to policymaking. Taken together, we suggest that a cascading framework of landscape structures, natural predators, invasive pests, and crop loss should be constructed to address the possible impacts of land-use change on sustainable agriculture in the future.

5. Conclusions

Landscape compositional heterogeneity with high forest cover was positively related to an invasive pest and its associator, whereas the negative effect of configurational heterogeneity on the invasive pest mainly depended on edge density. The relative effect of compositional heterogeneity on the invasive pest was lower than that of configurational heterogeneity, but the opposite was true for the associator. The direction of the relationships between landscape variables with the invasive pest and the associator did not change with spatial distance and combined scales, nor did the strengths of these relationships. Possible reasons for this are that species with wings have a high dispersal ability and habitat adaptability, and habitat types and landscape structure have limited variation at spatial scales. We believe that maintaining appropriate landscape fragmentation around crop patches is effective for inhibiting invasive pests, but further elucidation of the relationships between landscape effects, natural enemies, invasive pests, and ecological effects is necessary for developing agricultural conservation strategies in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

ZW designed the study, collected and analyzed data, and produced a draft of the manuscript. QY and BH provide technical support and process data. LZ, HZ, YS, YY, ZO, and RL provided comment on various drafts. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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EDITED BY
Fang Ouyang,
Institute of Zoology (CAS), China

REVIEWED BY
Huai-Jun Xue,
Nankai University, China
Yuzhe Du,
Agricultural Research Service (USDA),
United States
Jing Gao,
Institute of Zoology, Guangdong Academy of
Science (CAS), China

*CORRESPONDENCE
Yan Li Du
✉ yanlidu@126.com

†These authors share first authorship

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Bioactive volatile compounds from *Penicillium digitatum*-infected apples: Oviposition attractants for yellow peach moth *Conogethes punctiferalis* (Lepidoptera: Crambidae)

Hong Gang Guo†, Shuang Zhen Miao†, Peng Peng Ai,
Min Zhao Zhang, Zhe Yan and Yan Li Du*

College of Bioscience and Resource Environment/Key Laboratory of Urban Agriculture (North China),
Ministry of Agriculture and Rural Affairs of the People's Republic of China, Beijing University of Agriculture,
Beijing, China

Introduction: Plant-associated microbes critically shape the dynamics of plant- and insect-associated communities. In previous studies, we reported that the yellow peach moth *Conogethes punctiferalis* (YPM) preferred to *Penicillium digitatum-infected* apples (PDA) for oviposition. However, the underlying mechanisms remains unclear.

Methods: In the present study, the behavioral and physiological experiments were conducted to determine how *P. digitatum* affects the oviposition selection of mated YPM females *via* altering host plant volatile organic compounds (VOCs).

Results: Mated YPM females were attracted to and laid more eggs on PDA than on non-infected apples (NIA), mechanically damaged apples (MDA), and *P. digitatum* in potato dextrose agar medium (PPD) in the oviposition selection experiments. Four-arm olfactometer assays further confirmed that odors in PDA were responsible for the attractiveness of mated YPM females. Further analyses showed that 38 VOCs were collected and identified from all treatments by GC-MS, with five specific VOCs (methyl 2-methylbutyrate, styrene, methyl caproate, butyl caprylate, and *n*-tetradecane) emitting from PDA. A principal component analysis (PCA) based on the absolute contents of 38 VOCs revealed a clear separation of PDA from NIA, MDA, and PPD. Moreover, when *P. digitatum*-induced specific VOCs were added to apples in individual or synthetic blends, there was a significantly higher percentage of mated YPM females to apples with individual or synthetic blends consisting of methyl 2-methylbutyrate, butyl caprylate, or *n*-tetradecane in Y-tube olfactometer experiments, suggesting that these three specific VOCs acted as predominant olfactory signals for mated YPM females to PDA.

Discussion: Taken together, the microbe *P. digitatum* was an important driver of the interactions between YPMs and host plants by altering plant volatiles. These findings may form the basis for developing attractant baits for field trapping YPMs in the future.

KEYWORDS

***Conogethes punctiferalis*, *Penicillium digitatum*, plant-microbe-insect interactions, chemical communication, host plant volatile organic compounds**

Introduction

Plant-associated microbes are widely reported as important but overlooked drivers of host plant-herbivorous insect interactions, either direct effects of plant-associated microbes on herbivorous insects *via* the ingestion of microbes and/or microbial metabolites or indirect effects of plant-associated microbes *via* altering the host plant biochemistry (Eberl et al., 2018, 2019, 2020). The impacts of plant-associated microbes on herbivorous insects could further cascade up and down multiple trophic levels in the arthropod community at spatial scales ranging from patterns within single host plants to entire landscapes (Tack and Dicke, 2013). Consequently, it is meaningful to investigate tripartite interactions among plant-associated microbes, host plants, and herbivorous insects for improving our knowledge on the ecology and evolution of plant-microbe-insect interactions and designing more effective management strategies to control herbivorous insects in agroecosystems.

Plant-associated microbes exhibit diverse effects on the herbivorous insects' foraging behavior, such as the location and selection of host plants, including detrimental, beneficial, and neutral (Kopper et al., 2004; Witzgall et al., 2012). Furthermore, the changes in host plant volatile organic compounds (VOCs) caused by plant-associated microbes infection could be responsible for the alteration of herbivorous insects' foraging behavior (Groen et al., 2016; Rizvi et al., 2016; Grunseich et al., 2019). For example, the bacteria on the egg-surface, such as *Providencia* sp. and *Klebsiella* sp., increase the relative content of β-caryophyllene in host plant VOCs, and result in deterring the oviposition of *Bactrocera dorsalis* (Li et al., 2020). Interestingly, a recent study shows that *Lymantria dispar* L. are attracted to volatiles from rust spores (*Melampsora laricipopulina*) (Eberl et al., 2018), suggesting that VOCs emitted by both host plants and the microbes themselves are important for establishing tripartite interactions among herbivorous insects, host plants, and plant-associated microbes (Tasin et al., 2012; Fernandez-Conradi et al., 2018). Our previous results also found that mated yellow peach moth (*Conogethes punctiferalis*, YPM) females preferred to *Penicillium* fungi-infected apples, including *Penicillium sumatrense*-infected apples, *Penicillium citrinum*-infected apples, *Penicillium digitatum*-infected apples, and the components and proportions of apples' VOCs were changed by *Penicillium* fungi-infection (Shi et al., 2019; Guo et al., 2022). Furthermore, *P. digitatum*-infected apples were more attraction to mated YPM females than other two *Penicillium* fungi-infected apples, triggering us to explore which components of VOCs in *P. digitatum*-infected apples or *P. digitatum* itself were crucial for mediating the foraging preference of YPM females to *Penicillium*-infected apples.

The YPM is a generalist herbivorous insect and a serious pest in tropical and eastern Asia, and Australia. Damage caused by the tunneling of YPM larvae into fruits results in serious loss of apples, corns, chestnut, and other crops (Li et al., 2015; Du et al., 2016). Recent efforts for controlling this pest have been focused on modulating male behavior by sex pheromones (Xiao et al., 2012; Du et al., 2014). However, the current strategies of interrupting their normal mating with sex pheromones are useless for mated YPM females. Other strategies, including plant-derived attractants and/or repellents as allelochemicals that selectively manipulate the behavior of YPM females, demand for the integrated pest management of YPMs (Xiao et al., 2012; Luo and Honda, 2015a). *P. digitatum*, an important and common phytopathogen of citrus fruits in the postharvest period around the world, causes citrus green mold disease with the deterioration and rotting of citrus fruits (Bhatta, 2022). Furthermore, the orange-originated *P. digitatum* fungus could infect apple fruits with the typical symptom described in other studies (Shi et al., 2019), suggesting the infection capacity of the orange-originated *P. digitatum* fungus on apple fruits.

Therefore, further understanding the principle of chemical ecology about the effects of plant-associated microbes on YPMs might be meaningful for developing attractants based on bioactive host plant VOCs to trap YPM females. Keeping the above in view, we determined the effect of *P. digitatum* on the VOCs of apples, and the cascading effects on the host preference of mated YPM females. Our specific objectives were to determine (1) the oviposition selection and behavioral responses of YPM females among the non-infected apples (NIA), mechanically damaged apples (MDA), *P. digitatum*-infected apples (PDA), and *P. digitatum* in potato dextrose agar medium (PPD); (2) the differences of VOCs from NIA, MDA, PDA, as well as PPD; (3) which VOCs were key components for affecting oviposition behavior of YPMs.

Materials and methods

Insects

A colony of YPMs was established and had been maintained for about 25 generations on maize in climate incubators (RTOP-B, Zhejiang Top Instrument Co., Ltd.) at 23 ± 1°C, RH 75 ± 2%, 16L/8D photoperiod, and 3, 500 lux light intensity (Guo et al., 2021). Adult moths were provided with 5–8% honey solution after emergence. Apples covered with gauze pieces were provided for the oviposition of mated YPM females in the cage.

Fungal culture

The *P. digitatum* isolated from orange fruits were purified using the traditional tissue separation method (Shi et al., 2019). The symptomatic–asymptomatic junction tissue was cut into segments (about 0.5 × 0.5 cm^2), which was immersed completely into 2% sodium hypochlorite for 3 min and flushed with sterilized water three times, and then were immersed completely into 75% ethanol for 1 min and flushed with sterilized water for three times. Finally, the segments were incubated onto potato dextrose agar (containing (g/L): potato 200; dextrose 20; agar 18) medium in Petri dishes and placed in a constant temperature incubator at 28°C. After repeated purification for four times, the pure culture of *P. digitatum* was obtained. Potato dextrose agar medium (7 mm diameter) with fully grown *P. digitatum* (PPD) was also prepared and incubated at 28°C for 6 d before behavioral assays.

Reparation of conidial suspension

The *P. digitatum* was cultured on potato dextrose agar at 25°C to prepare the conidial suspension. The potato dextrose agar culture of *P. digitatum* bearing 7-d-old conidia was gently rinsed in 1.5 mL sterilized distilled water and the density of conidial suspension was adjusted to 8×10^7 conidia/mL.

Apple treatments

Non-infected apples

Apple (*Malus pumila*, Red fuji variety) fruits with uniform size (7–9 cm diameter) and shape were bought from the supermarket of Beijing University of Agriculture (Beijing, China) and were stored at 4°C in a refrigerator. Apple fruits with uniform size were firstly sterilized using 75% alcohol for 1 min, and then 1% sodium hypochlorite for 3 min, at last washed with sterilized distilled water under the horizontal-laminar airflow clean bench for further experiments.

Mechanically damaged apples

Apple fruits with uniform size were sterilized as NIA treatment. Two holes (7 mm diameter) at the opposite sides of each apple were punched and immediately stuffed using sterilized fungus-free potato dextrose agar medium.

P. digitatum-infected apples

Apple fruits with uniform size were sterilized as NIA treatment. Two holes were punched as MDA, and then stuffed using potato dextrose agar medium with fully grown *P. digitatum*.

After treatments, each apple was placed into a sterilized plastic box (25 × 18× 12 cm) and incubated at 28°C for 2, 4, 6, and 8 d before used for following behavioral assays. Considering to the fact that the apples infected by *P. digitatum* for 8 d or longer would become rotten in the following days, the apples infected for 6 d were, therefore, used in the later experiments.

Oviposition behavioral experiments

To test the effects of *P. digitatum* on the oviposition behavior of mated YPM females, four treatments (NIA, MDA, PDA, and PPD) were simultaneously offered in a wood-frame cage (35 × 27 × 25 cm) with plastic gauzes on side walls to allow the oviposition of mated YPM females. The experimental materials (NIA, MDA, PDA, and PPD) were individually put into two opposite plastic bowls that were punched holes in advance. The materials were renewed every day and their positions were randomly changed. Ten newly emerged naive females and 15 males (no exposure to natural or synthetic sources of apples or fungi-infected apples) had been released into a cage to copulate for 3–4 days before the oviposition selection experiments. Each experiment was replicated 20 times with a total number of 200 females. The egg numbers on each sheet were counted separately and the data were statistically treated on the basis of average number of eggs by 10 females.

Four-arm olfactometer experiments

Four-arm olfactometer was used to test two bioassay experiments about the behavioral responses of mated YPM females, one is to the odors from NIA, MDA, PDA, and PPD, the other is to different concentrations (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} (v/V)) of the same specific VOCs from *P. digitatum*-infected apples. According to our previous study, the four-arm olfactometer consists of four glass chambers (2.5 cm diameter, 10.0 cm long), each with terminal end coupling to a cap (3.0-cm-long arms, 2.0 cm diameter) and central converging into a 10-cm-long common arm (2.5 cm diameter) (Shi et al., 2019). Moistened and charcoal-filtered air was pumped through each odor and then respectively went into one of the caps at a rate of 250 ml/min controlled by flow meters (LZYIA Instrument Co. Ltd, China). One moth was introduced into the entrance of the common arm of the olfactometer using a glass vial, and its behavioral response was observed under a 25-W red light lamp. The test for each moth lasted 3 min, and the behavioral response was classified as a choice if the moth passed over 1/3 length of the arm associated with one of the four odors and stayed there for more than 30 s. Conversely, no-choice was assigned if the test moth remained in the common arm for 3 min. The position of the lateral chambers along with the olfactometer was systematically exchanged after testing 2 moths to avoid positional bias. The olfactometer was flushed following Du et al. (2016). The moths used for test were allowed to acclimatize to the test conditions for 2 h before the start of the test. 3- to 4-day-old mated females were used and each individual moth was used only once. The selection rate in the four-arm olfactometer experiment was defined as the number of females that made a selection for the odor divided by the total number of females that made a selection for any odors offered simultaneously.

The experiment 1: the behavioral responses of mated YPM females to the odors from NIA, MDA, PDA, and PPD. Three apples of each treatment (NIA, MDA, PDA) and 4 pieces of PPD (diameter 7 mm) were placed into an oven bag (Reynolds Kitchens, Richmond, VA, USA) respectively. Each oven bag was tightened and connected with a Teflon tube to one of the caps of the four-arm olfactometer for behavioral tests. A total of 211 individual mated YPM females were tested.

The experiment 2: the behavioral responses of mated YPM females to different concentrations of the five specific VOCs from *P. digitatum*-infeced apples. For each VOC, four concentrations (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} (v/V)) were prepared with mineral oil as solvent. And then, an aliquot of 10 μl test solution for each of the four concentrations of the same compound was applied onto a 1 × 5 cm filter paper, which was thereafter placed into one chamber of the four-arm olfactometer for behavioral tests. Totally, 80 mated YPM females (styrene), 86 mated YPM females (methyl 2-methylbutyrate), 80 mated YPM females (methyl caproate), 85 mated YPM females (butyl caprylate), and 80 mated YPM females (*n*-tetradecane) were tested.

All chemicals (purities ≥ 95%) were purchased from commercial companies, which methyl 2-methyl butyrate and styrene were from J & K Chemical Ltd. (Shanghai, China), methyl caproate, butyl caprylate, and *n*-tetradecane from TCI Development Co., Ltd. (Shanghai, China).

VOCs collection and analysis

Five apples of each treatment (NIA, MDA, PDA) and correspondingly similar size of PPD that were placed into a 48.2 × 59.6 cm oven bag respectively were used to collected VOCs according to dynamic headspace collection method reported by Guo et al. (2021).

The bag mouth was tightened with a twist tie around a glass tube (6 mm diameter, 10 cm long) filled with 50 mg of Porapark Q adsorbent (80–100 mesh, Waters Corporation). The humidified and purified air was pushed at a rate of 450 ml/min by a QC-1S pump (Labor Protection Science Research Institute of Beijing) into the bag through the Teflon tube and was pulled out through the glass tube filled with Porapark Q adsorbent. VOCs were trapped by the Porapark Q adsorbent when they passed through the glass tube.

After collection, the trapped VOCs were eluted using chromatography-grade *n*-hexane (99.9%) and then were analyzed using an Agilent 6,890 gas chromatograph (GC) coupled to an Agilent 5,975 Mass Spectrometer (MS). The procedures for the GC-MS analysis were the same as described in Du et al. (2016) with the exceptions that the GC was equipped with a DB-5MS column (60 m × 0.25 mm × 0.15 µm, Agilent, USA) rather than a HP-5MS column (30 m × 0.25 mm × 0.25 µm) and the injector temperature was 250°C other than 210°C. Following injection, the column temperature was maintained at 37°C for 6 min, followed by an increase in temperature of 2°C/min to 70°C for 5 min, and then an increase of 5°C/min up to 200°C, at last maintained at 200°C for 5 min. Compounds were tentatively identified by comparing mass spectra with NIST Standard Reference Database 98 (Agilent Technologies, Palo Alto, CA, USA). Compounds were quantified by their total ion abundance relative to that of the internal standard (*n*-nonyl acetate).

Electroantennogram assays

Five specific VOCs from PDA, including methyl 2-methyl butyrate, styrene, methyl caproate, butyl caprylate, and *n*-tetradecane, were chosen for electroantennogram (EAG) measurements. All chemicals (purities ≥ 95%) were purchased from commercial companies, which methyl 2-methyl butyrate and styrene were from J & K Chemical Ltd. (Shanghai, China), methyl caproate, butyl caprylate, and *n*-tetradecane from TCI Development Co., Ltd. (Shanghai, China). Four concentrations [10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} (v/V)] of five individual compounds were prepared as the four-arm olfactometer experiment 2. The test solutions were stored at −20°C for further EAG analyses.

EAG recordings were performed on 3- to 4-day-old mated YPM females that the moths at this stage were eggs-loading and sensitive to signals used for oviposition location (Belmain et al., 2002). The method of EAG recordings was the same as that described by Du et al. (2016). Stimulus was delivered and tested in increasing doses on the antennae of mated YPM females with mineral oil and *n*-hexanol being used as control and standard stimuli, respectively. EAG test was run for a variable number of replicates per day, and each compound at each concentration was tested on 15 antennae. In each test, the control and standard stimuli were applied subsequently after four successive stimulations. Normalization was achieved by dividing the peak EAG amplitude of the test puff with the average EAG amplitude of the two nearest standard stimulations after subtracting the amplitude recorded in response to the mineral oil.

Y-tube olfactometer experiments

The preference of mated YPM females to apples with or without five specific VOCs (methyl 2-methylbutyrate, styrene, methyl caproate, butyl caprylate, and *n*-tetradecane) from PDA was tested using Y-tube olfactometer. For Y-tube olfactometer assays, apples with and without five specific VOCs from PDA [the amount was referred to the concentrations in fungi-infected apple fruits (Supplementary Table S1)] were separately placed into the chambers of the Y-tube. The test procedure was similar to that in our previous study (Guo et al., 2022). Each individual moth was used only once, and totally 80 mated female moths were tested for each treatment. The selection rate in the Y-tube olfactometer experiment was defined as the number of females that made a selection for apples with exogenous compounds divided by the total number of females that made a selection between apples with and without exogenous compounds.

Statistics analyses

Data obtained from oviposition selection experiments, behavioral assay in four-arm olfactometers, EAG tests, and the absolute content of host plant VOCs were subjected to analysis of variance (ANOVA) using Tukey-HSD test ($P < 0.05$). The data of Y-tube olfactometer experiments were analyzed using non-parametric *Chi*-square analysis (Females with no choice were excluded from statistical analyses, $P < 0.05$). The quantification of VOCs measured as the absolute content of each compound was analyzed using principal component analysis (PCA) by the software program SIMCA P+ 11.0 (Umetrics AB, Umeå, Sweden) ($P < 0.05$). All statistics except the PCA analysis were performed using the SPSS16.0 statistical software. Graphs were generated in the program of Graphpad Prism 9.0.

Results

Oviposition selection of YPMs among NIA, MDA, PDA, and PPD

In order to assess the effects of *P. digitatum* on the oviposition selection of mated YPM females, the apples infected with *P. digitatum* for 2, 4, 6, and 8 d were simultaneously provided to allow the oviposition selection of mated YPM females. The results showed that the number of eggs on the 8d-PDA was significantly higher than those on the 2d- and 4d-PDA, and was larger (but not significant) than that on the 6d-PDA ($F_{3,\,79} = 11.699$, $P < 0.01$; Figure 1A). When NIA, MDA, PDA, and PPD were simultaneously offered in a cage to allow for oviposition, the average number of eggs laid by 10 mated YPM females (173.2) was significantly higher on PDA than those on MDA (22.3), NIA (22.2), and PPD (5.7) ($F_{3,\,79} = 111.413$, $P < 0.01$; Figure 1B), respectively.

Selection behavior of YPMs among odors of NIA, MDA, PDA, and PPD

The behavioral responses of mated YPM females were tested in a four-arm olfactometer. The selection rate of mated YPM females to the odor of PDA (42.03%) was significantly higher than those to the odor of NIA (17.91%), the odor of MDA (24.17%), and the odor of PPD (15.89%) ($F_{3,19} = 18.948$, $P < 0.01$; Figure 1C), suggesting that mated YPM females preferred to the odor of PDA.

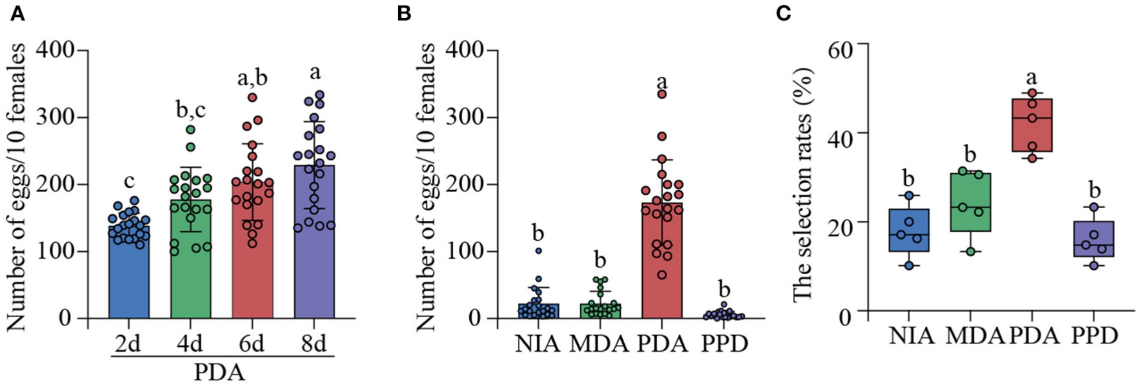

FIGURE 1
The selection behavior of mated YPM females. **(A)** The average egg numbers of mated YPM females on *P. digitatum*-infected apples among different infection time (2, 4, 6, and 8 d, respectively). It was repeated five times. **(B)** The average egg numbers of mated YPM females among PPD, NIA, MDA, and PDA. It was repeated five times. **(C)** The selection rates of four-arm olfactometer experiments among the odors from PPD, NIA, MDA, and PDA. A total of 211 mated YPM females were tested for four-arm olfactometer experiments. Different letters indicated significant difference among different treatments (Tukey-HSD test after ANOVA, $P < 0.05$). Non-infected apples (NIA), Mechanically damaged apples (MDA), *P. digitatum*-infected apples (PDA), and *P. digitatum* in potato dextrose agar medium (PPD).

VOCs profiles

In total, 38 volatile compounds were detected from the emissions of NIA, MDA, PDA, and PPD, including 24 compounds in NIA, 22 compounds in MDA, 24 compounds in PDA, and 1 compound in PPD (Supplementary Figure S1; Table 1). The results showed that α-farnesene was the most abundant compound in NIA, MDA, and PDA. Compared with MDA and NIA, five compounds, including methyl 2-methylbutyrate, styrene, methyl caproate, butyl caprylate, and *n*-tetradecane, were specifically detected in PDA. Only one compound, ethyl butyrate, was found in PPD (Table 1).

A principal component analysis (PCA) based on the absolute content of the 38 compounds was then performed to determine the major sources of variations in the four treatments (NIA, MDA, PDA, and PPD). The biplot depicted by graphical PCA expounded the first two principal components (PCs) with an explication of 70.61% of the total variance in the four treatments. The first component (PC1), which explained 50.68% of the total variance, was clearly isolated NIA. The second component (PC2) accounted for 19.93% of total variance (Figure 2A). Further analyses indicated that the major loadings of PC1 were for *n*-butyl butyrate (-0.995), isoamyl 2-methylbutyrate (−0.995), hexyl hexanoate (-0.989), amyl hexanoate (-0.989) and major loadings of PC2 were for styrene (0.887), ethyl octanoate (0.887), α-farnesene (0.868), ethyl caproate (0.865). And then, PC scores were subjected to one-way ANOVA. The results showed that NIA was significantly higher than PDA and MDA in PC1 scores, and PDA had the highest PC2 scores among four treatments (Figures 2B, C).

EAG and behavioral responses of mated YPM females to volatile compounds

Five specific VOCs emitted from PDA, including methyl 2-methylbutyrate, styrene, methyl caproate, butyl caprylate, and *n*-tetradecane, were selected for EAG and behavioral tests. Each of the five synthetic compounds could trigger significant EAG responses of mated YPM females, with dose-dependent decreasing from 10^{-1} to 10^{-4} (v/V) (Figure 3). Of which, the strongest EAG response was elicited by methyl caproate at 10^{-1} (v/V) (EAG value = 1.314 μA) (Figure 3C), followed by methyl 2-methylbutyrate at 10^{-1} (v/V) (EAG value = 0.995 μA) (Figure 3B) and butyl caprylate at 10^{-1} (v/V) (EAG value = 0.790 μA) (Figure 3D). The compound methyl 2-methylbutyrate at concentration of 10^{-2} (v/V) (EAG value = 0.620 μA) (Figure 3B) could trigger stronger EAG responses than styrene at 10^{-1} (v/V) (EAG value = 0.602 μA) (Figure 3A) and, butyl caprylate at 10^{-2} (v/V) (EAG value = 0.398 μA) (Figure 3D) and styrene at 10^{-2} (v/V) (EAG value = 0.374 μA) (Figure 3A) both trigger stronger EAG responses than *n*-tetradecane at 10^{-1}(v/V) (EAG value = 0.347 μA) (Figure 3E).

To further predict the optimal concentration for attraction, the four-arm olfactometer was further used to determine the simultaneous selection of *C. punctiferalis* mated females among four concentrations [10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} (v/V)] of each same compound. The behavioral responses indicated that the five individual compounds all showed the strongest attractiveness to the mated YPM females at 10^{-2} (v/V) in the four-arm olfactometer experiments (Figure 4). For styrene, the selection rates of mated YPM females were 35.89% at 10^{-3} (v/V) and 39.56% at 10^{-2}(v/V), which was significantly higher than those of 14.19% at 10^{-4} (v/V) and 10.35% at 10^{-1} (v/V) (Figure 4A). The selection rate of mated YPM females was highest at 10^{-2} (v/V) (41.82%) among four concentrations of methy1 2-methylbutyrate, followed by those at 10^{-1} (v/V) (25.58%) and 10^{-4} (v/V) (17.48%), and the lowest was at 10^{-3} (v/V) (15.13%) (Figure 4B). The selection rate of mated YPM females was significantly higher at 10^{-2} (v/V) (34.24% of methyl caproate and 36.97% of *n*-tetradecane) than those at 10^{-1} (v/V) (13.64% of methyl caproate and 17.79% of *n*-tetradecane), while it was intermediate at 10^{-3} (v/V) (24.72% of methyl caproate and 24.55% of *n*-tetradecane) and 10^{-4} (v/V) (27.39% of methyl caproate and 20.69% of *n*-tetradecane) (Figures 4C, E). For butyl caprylate, the selection rate of mated YPM females at 10^{-2} (v/V) (37.88%) was the highest, followed by those at 10^{-3} (v/V) (24.24%), 10^{-4} (v/V) (19.70%), and 10^{-1} (v/V) (16.67%) (Figure 4D).

TABLE 1 VOCs were identified[a] in headspace collections from four treatments[b].

No.	Compounds	CAS	Retention time (min)	Relative content (%)[c]				Absolute content (μg/μL)[d]			
				NIA	MDA	PDA	PPD	NIA	MDA	PDA	PPD
1	Ethyl propionate	105-37-3	4.36	—	2.63 ± 1.17^{3}	—	—	—	2.75 ± 1.36	—	—
2	*n*-Propyl acetate	109-60-4	4.43	—	0.36^{1}	—	—	—	0.39	—	—
3	2-Methyl-1-butanol	137-32-6	5.10	—	0.94 ± 0.27^{2}	0.43 ± 0.11^{3}	—	—	0.74 ± 0.01	0.58 ± 0.12	—
4	Methyl 2-methylbutyrate	868-57-5	6.34	—	—	0.39 ± 0.10^{4}	—	—	—	0.56 ± 0.05	—
5	Ethyl butyrate	105-54-4	7.56	—	9.57 ± 2.19^{3}	2.68 ± 0.22^{5}	100.00 ± 4.17^{3}	—	10.58 ± 3.95^{b}	3.94 ± 0.42^{ab}	0.59 ± 0.08^{a}
6	*n*-Propyl propionate	106-36-5	7.98	—	0.35 ± 0.05^{2}	—	—	—	0.42 ± 0.02	—	—
7	*n*-Butyl acetate	123-86-4	8.20	2.63 ± 0.31^{3}	3.18 ± 0.47^{3}	1.01 ± 0.24^{5}	—	5.08 ± 1.05^{b}	3.02 ± 0.25^{b}	1.47 ± 0.40^{a}	—
8	Ethyl-2-methylbutyrate	7452-79-1	10.27	—	21.44 ± 2.45^{3}	13.86 ± 0.55^{5}	—	—	21.87 ± 5.08	21.28 ± 3.17	—
9	2-Methybutyl acetate	624-41-9	12.21	9.34 ± 0.65^{3}	11.53 ± 1.75^{3}	5.17 ± 0.64^{5}	—	17.74 ± 2.14^{b}	11.01 ± 1.19^{b}	7.78 ± 1.39^{a}	—
10	Styrene	100-42-5	12.58	—	—	2.08 ± 0.55^{5}	—	—	—	3.32 ± 1.06	—
11	*n*-Propyl butyrate	105-66-8	13.37	0.66 ± 0.11^{3}	0.32 ± 0.01^{2}	—	—	1.29 ± 0.32	0.39 ± 0.03	—	—
12	Butyl propionate	590-01-2	14.12	1.07 ± 0.27^{3}	0.36 ± 0.01^{2}	—	—	2.11 ± 0.66	0.43 ± 0.05	—	—
13	Amyl acetate	628-63-7	14.48	0.47 ± 0.03^{2}	0.65 ± 0.01^{2}	0.35^{1}	—	0.95 ± 0.14	0.79 ± 0.08	0.64	—
14	Methyl caproate	106-70-7	15.16	—	—	0.44 ± 0.03^{2}	—	—	—	0.81 ± 0.05	—
15	Ethyl tiglate	5837-78-5	16.18	—	0.42^{1}	—	—	—	0.46	—	—
16	Propyl 2-methylbutyrate	37064-20-3	16.72	0.68 ± 0.07^{3}	1.23 ± 0.36^{3}	0.96 ± 0.13^{5}	—	1.30 ± 0.21^{a}	1.11 ± 0.09^{a}	1.49 ± 0.32^{a}	—
17	*n*-Butyl butyrate	109-21-7	20.62	2.89 ± 0.43^{3}	—	—	—	5.59 ± 1.21	—	—	—
18	Ethyl caproate	123-66-0	20.79	0.35 ± 0.06^{2}	4.32 ± 0.94^{3}	5.74 ± 0.70^{5}	—	0.60 ± 0.10^{a}	4.75 ± 2.90^{b}	8.94 ± 1.80^{b}	—
19	Hexyl acetate	142-92-7	21.91	4.21 ± 0.61^{3}	3.71 ± 0.26^{3}	2.88 ± 0.19^{5}	—	8.15 ± 1.82^{a}	3.85 ± 1.00^{a}	4.28 ± 0.51^{a}	—
20	Butyl 2-methylbutyrate	15706-73-7	24.09	2.75 ± 0.45^{3}	1.16 ± 0.34^{3}	0.97 ± 0.11^{5}	—	5.30 ± 1.11^{b}	1.05 ± 0.12^{a}	1.41 ± 0.16^{a}	—
21	2-Methylbutyl butyrate	51115-64-1	25.60	0.53 ± 0.02^{2}	—	—	—	1.07 ± 0.13	—		—
22	Propyl caproate	626-77-7	29.22	1.58 ± 0.04^{3}	—	0.48 ± 0.04^{4}	—	2.99 ± 0.25	—	0.81 ± 0.10	—
23	2-Methylbutyl 2-Methylbutyrate	2445-75-8	29.81	0.75 ± 0.09^{3}	—	0.55 ± 0.04^{2}	—	1.44 ± 0.24	—	0.97 ± 0.11	—
24	Hexyl propionate	2445-76-3	30.06	0.80 ± 0.20^{3}	—	—	—	1.58 ± 0.50	—		—
25	Isoamyl 2-methylbutyrate	27625-35-0	32.14	0.37 ± 0.05^{3}	—	—	—	0.71 ± 0.13	—		—
26	Hexyl isobutyrate	2349-07-7	32.73	0.27 ± 0.03^{2}	—	—	—	0.53 ± 0.00	—		—
27	Butyl hexanoate	626-82-4	35.09	11.57 ± 0.34^{3}	0.96 ± 0.12^{3}	1.42 ± 0.22^{5}	—	22.00 ± 2.51^{b}	0.94 ± 0.20^{a}	1.99 ± 0.14^{a}	—
28	Ethyl octanoate	106-32-1	35.16	—	0.82 ± 0.17^{3}	1.63 ± 0.36^{5}	—		0.88 ± 0.33	2.86 ± 0.69	—

(Continued)

TABLE 1 (Continued)

No.	Compounds	CAS	Retention time (min)	Relative content (%)^c				Absolute content (μg/μL)^d			
				NIA	MDA	PDA	PPD	NIA	MDA	PDA	PPD
29	Hexyl 2-methylbutyrate	10032-15-2	36.88	11.91 ± 0.95^3	4.34 ± 0.81^3	6.09 ± 1.10^5	—	22.74 ± 3.34^b	4.12 ± 0.55^a	9.00 ± 2.00^a	—
30	2-Methylbutyl hexanoate	2601-13-0	37.40	1.50 ± 0.06^3	—	0.39 ± 0.04^3	—	2.83 ± 0.21	—	0.57 ± 0.11	—
31	Amyl hexanoate	540-07-8	38.68	1.07 ± 0.05^3	—	—	—	2.03 ± 0.26	—	—	—
32	Propyl octanoate	624-13-5	38.79	0.28 ± 0.03^3	—	—	—	0.52 ± 0.03	—	—	—
33	Ethyl trans-4-decenoate	76649-16-6	41.51	—	0.32^1	0.74 ± 0.06^5	—	—	0.42	0.97 ± 0.15	—
34	Hexyl hexanoate	6378-65-0	41.78	6.35 ± 0.34^3	0.38 ± 0.07^2	0.79 ± 0.20^5	—	12.12 ± 1.67^b	0.47 ± 0.13^a	1.31 ± 0.33^a	—
35	Butyl caprylate	589-75-3	41.78	—	—	0.27 ± 0.03^2	—	—	—	0.42 ± 0.02	—
36	*n*-Tetradecane	629-59-4	42.16	—	—	0.24 ± 0.04^3	—	—	—	0.40 ± 0.02	—
37	Isoamyl caprylate	2035-99-6	43.49	0.36 ± 0.01^3	—	—	—	0.69 ± 0.07	—	—	—
38	α-Farnesene	502-61-4	45.20	36.55 ± 5.01^3	30.57 ± 2.52^3	49.24 ± 1.02^5	—	67.88 ± 5.40^a	30.57 ± 6.36^a	74.25 ± 9.11^b	—

[a] Identity of compounds was confirmed by comparison of mass spectrum and retention time with those of the respective synthetic standards.

[b] NIA, non-infected apples; MDA, mechanically damaged apples; PPD, P. digitatum-infected apples; PDA, P. digitatum in potato dextrose agar medium.

[c] Values are mean ± SE (n = 2–5). The superscript numbers of mean values indicated the times that the compound was identified in different repetitions of the same treatment.

[d] Values are mean ± SE (n = 2–5). Different letters within the same row indicated the significant difference among four treatments (P < 0.05).

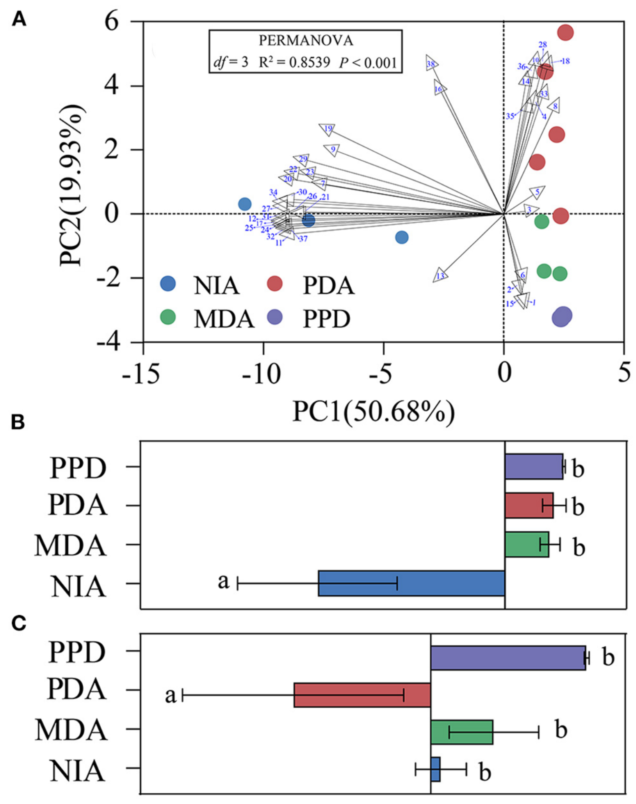

FIGURE 2
Principal component analysis based on 38 compounds which were obtained from NIA, MDA, PDA, and PPD. **(A)** The percentage of total variance explained by the first two principal components (PCs). **(B)** The overall distribution of PC1 scores within four treatments. **(C)** The overall distribution of PC2 scores within four treatments. Different letters indicated significant difference among four treatments (Tukey-HSD test after ANOVA, $P < 0.05$). The numbers in the graph were the same as those in Table 1. Non-infected apples (NIA), Mechanically damaged apples (MDA), *P. digitatum*-infected apples (PDA), and *P. digitatum* in potato dextrose agar medium (PPD). The VOCs of PDA were identified in five repetitions. The VOCs of NIA, MDA, PPD were identified in three repetitions.

To address the critical synergist in attracting mated YPM females to fungi-infected apples, the individual or mixed blends of five specific VOCs were added onto the mechanically damaged apples (MDA) respectively to test the behavioral responses, with MDA as control (Figure 5; Supplementary Table S1). For individual compounds, mated YPM females preferred to apples with methyl 2-methylbutyrate (the selection rate of 66.25%, $\chi^2 = 8.45$, $P = 0.004$) (Figure 5B), butyl caprylate (the selection rate of 61.25%, $\chi^2 = 4.05$, $P = 0.044$) (Figure 5D), and *n*-tetradecane (the selection rate of 66.25%, $\chi^2 = 8.45$, $P = 0.004$) (Figure 5E). However, mated YPM females had significant repellence to apples with styrene (the selection rate of 37.5%, $\chi^2 = 5.00$, $P = 0.025$) (Figure 5A). For mixed compounds, mated YPM females had significant preference to apples with methyl 2-methylbutyrate and butyl caprylate (the selection rate of 62.5%, $\chi^2 = 5.00$, $P = 0.025$) (Figure 5K), methyl 2-methylbutyrate and *n*-tetradecane (the selection rate of 63.75%, $\chi^2 = 6.05$, $P = 0.014$) (Figure 5L), butyl caprylate and *n*-tetradecane (the selection rate of 70%, $\chi^2 = 12.8$, $P < 0.001$) (Figure 5O), styrene, methyl 2-methylbutyrate, and *n*-tetradecane (the selection rate of

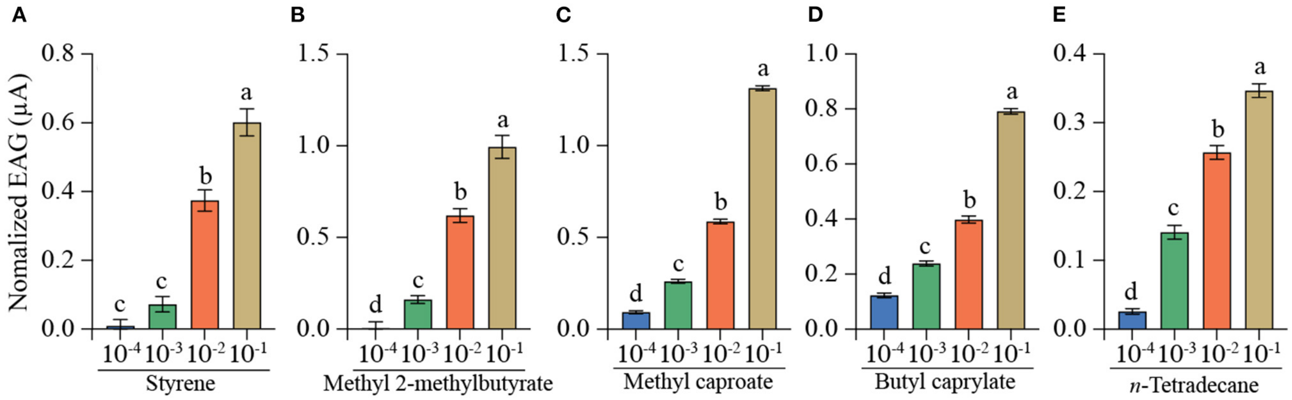

FIGURE 3
EAG responses of mated YPM females to five individual compounds. Bars represented mean ± SE ($n = 15$). Different letters on the bars indicated significant difference among the four concentrations of the same compounds (Tukey-HSD test after ANOVA, $P < 0.05$). **(A)** styrene, **(B)** methyl 2-methylbutyrate, **(C)** methyl caproate, **(D)** butyl caprylate, **(E)** *n*-tetradecane. Each compound at each concentration was tested on 15 antennae, and a total of 300 antennae were tested for all treatments.

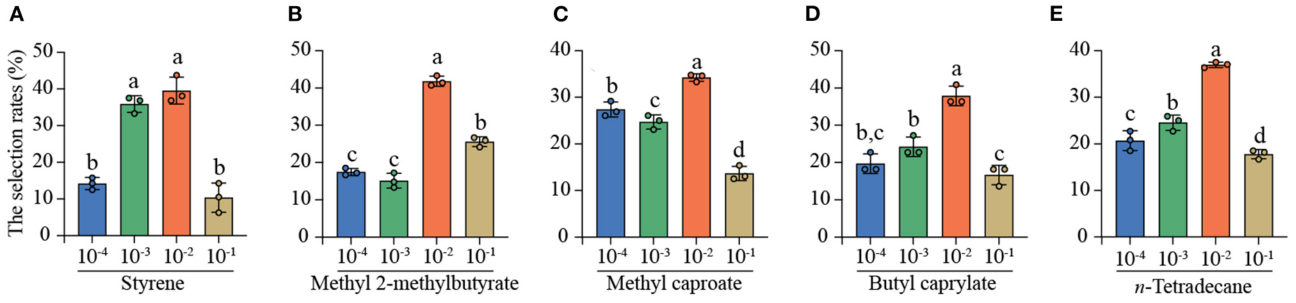

FIGURE 4
The selection rates of mated YPM females among the four concentrations [10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} (v/V)] of the same compounds in four-arm olfactometer. Different letters on the bars indicated significant difference among the four concentrations of the same compounds (Tukey-HSD test after ANOVA, $P < 0.05$). **(A)** styrene, **(B)** methyl 2-methylbutyrate, **(C)** methyl caproate, **(D)** butyl caprylate, **(E)** *n*-tetradecane. 80 mated YPM females (styrene), 86 mated YPM females (methyl 2-methylbutyrate), 80 mated YPM females (methyl caproate), 85 mated YPM females (butyl caprylate), and 80 mated YPM females (*n*-tetradecane) were tested.

62.5%, $\chi^2 = 5.00$, $P = 0.025$) (Figure 5R), methyl 2-methylbutyrate, methyl caproate, and butyl caprylate (the selection rate of 67.5%, $\chi^2 = 9.80$, $P = 0.002$) (Figure 5V), styrene, methyl 2-methylbutyrate, methyl caproate, and butyl caprylate (the selection rate of 61.25%, $\chi^2 = 4.05$, $P = 0.044$) (Figure 5Z), methyl 2-methylbutyrate, methyl caproate, butyl caprylate, and *n*-tetradecane (the selection rate of 61.25%, $\chi^2 = 4.05$, $P = 0.044$) (Figure 5AD), styrene, methyl 2-methylbutyrate, methyl caproate, butyl caprylate, and *n*-tetradecane (the selection rate of 63.75%, $\chi^2 = 6.05$, $P = 0.014$) (Figure 5AE).

Discussion

Plant-associated microbes and herbivorous insects often co-occur on the same host plant. It has indicated that plant-associated microbes have significantly cascading effects on host preference of herbivorous insects *via* affecting host plant VOCs (Fernandez-Conradi et al., 2018; Grunseich et al., 2019). In the present study, *P. digitatum* infection changed the VOCs profile of apple fruits, including five specific VOCs that were methyl 2-methylbutyrate, styrene, methyl caproate, butyl caprylate, and *n*-tetradecane, and consequently attracted the oviposition of YPM females. These findings implied that the roles of plant-associated microbes should be taken into account in the interactions between YPMs and host plants.

Plant associated microbes could alter the oviposition and foraging behaviors of subsequent herbivorous insects. However, there are no uniform effects of plant-associated microbes on insects' behavior. For example, *Botrytis cinerea* has an avoidance response of *Lobesia botrana* for laying eggs on the grape plants (Tasin et al., 2012). On the contrary, YPM females laid more eggs on PDA than on NIA and MDA, as well as than on PPD in the current study. Furthermore, the number of eggs laid by YPM females increased along *P. digitatum* infection time. The positive effects of plant-associated microbes on the preference of herbivorous insects for host plants have also been reported in other studies (Cardoza et al., 2002, 2003), confirming the important roles of plant-associated microbes in the host selection of herbivorous insects. Moreover, a meta-analysis of 1,113 case studies gathered from 101 primary papers suggests that the concept of tripartite interactions among host plants, plant-associated microbes, and herbivorous insects is dependent on microbes lifestyle (biotrophic or necrotrophic pathogens), herbivorous insects feeding guild (sap-sucking or chewing insects), and the spatial scale of

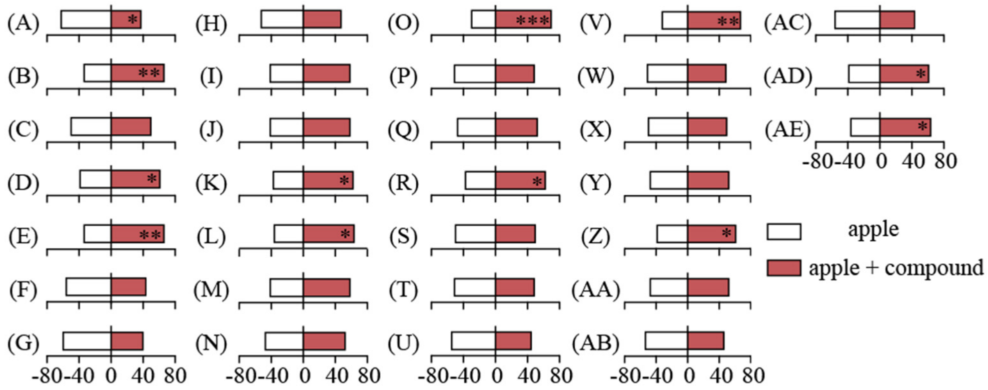

FIGURE 5

Behavioral responses of mated YPM females to apples with and without compounds in Y-tube olfactometer. The bars represented the percentage of the responding mated YPM females to apples with or without compounds. Stars indicated significant difference in Y-tube olfactometer assays using χ^2 test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). 1. Individual compound: **(A)** compound 1, **(B)** compound 2, **(C)** compound 3, **(D)** compound 4; **(E)** compound 5; 2. compounds' mixture: **(F)** compounds (1+5); **(G)** compounds (1+2); **(H)** compounds (1+4); **(I)** compounds (1+3); **(J)** compounds (2+3); **(K)** compounds (2+4); **(L)** compounds (2+5); **(M)** compounds (3+4); **(N)** compounds (3+5); **(O)** compounds (4+5); 3. compounds' mixture: **(P)** compounds (1+2+3); **(Q)** compounds (1+2+4); **(R)** compounds (1+2+5); **(S)** compounds (1+3+4); **(T)** compounds (1+3+5); **(U)** compounds (1+4+5); **(V)** compounds (2+3+4); **(W)** compounds (2+3+5); **(X)** compounds (2+4+5); **(Y)** compounds (3+4+5); 4. compounds' mixture: **(Z)** compounds (1+2+3+4); **(AA)** compounds (1+2+3+5); **(AB)** compounds (1+2+4+5); **(AC)** compounds (1+3+4+5); **(AD)** compounds (2+3+4+5); 5. compounds' mixture: **(AE)** compounds (1+2+3+4+5). Compound 1: styrene; Compound 2: methyl 2-methylbutyrate; Compound 3: methyl caproate; Compound 4: butyl caprylate; Compound 5: *n*-tetradecane. 80 mated YPM females were tested for each compound, and a total of 2,480 mated YPM females were tested for all compounds.

the interaction (local or distant) (Fernandez-Conradi et al., 2018), suggesting that different factors remain to be explored in the overall effects of *P. digitatum* on the performance and host preference of YPMs in the further experiments.

Chemical communication is an ancient and ubiquitous channel to mediate species interactions, and host plant VOCs is defined as olfactory cues in host location, recognition and selection of herbivorous insects. The YPM females were attracted to and laid eggs on artificial substrates that released host plant odors (Luo and Honda, 2015a,b; Du et al., 2016). In the present study, the VOCs profile of PDA was significantly different from NIA, MDA and PPD. Furthermore, mated YPM females had an obvious preference for PDA odors to NIA, MDA, and PPD odors in the four-arm olfactometer, which was not only in line with the oviposition preference of YPM females for PDA, but also further indicated the potential role of host plant VOCs for the preference of YPM females. Moreover, our experiments revealed a clear separation between VOC profiles of PDA (including five *P. digitatum*-induced specific VOCs) vs. MDA or NIA *via* PCA analysis, implying that these specific VOCs could be served as signals for the oviposition and foraging behaviors of YPMs to PDA. These results are agree with recent studies that host plant VOCs could be frequently altered by plant-associated microbes' infection and consequently have impacts on the host selection of herbivorous insects (Groen et al., 2016; Rizvi et al., 2016; Grunseich et al., 2019). Thus, it is not uncommon for herbivorous insects to employ fungi-induced kairomones for host location.

Some specific VOCs are emerged as attractants in the oviposition and foraging behavior of herbivorous insects (Turlings and Erb, 2018). For example, styrene is reported to be a spoilage marker of decayed apples after infection by *Penicillium expansum* and elicits strong electrophysiological antennal activity for *Ips typographus* at very low levels (Kim et al., 2018, 2019; Schiebe et al., 2019). As in our study, styrene was specifically emitted in a relatively ample amount from PDA and triggered significant EAG responses. However, mated YPM females showed obvious repellence to the styrene-supplemented apples in Y-tube olfactometer assay, which is the same as a previous study that styrene significantly reduces pine weevils' attraction to cut pieces of Scots pine twigs (Azeem et al., 2013). Indeed, YPM females exhibited significant preference to apples with three other *P. digitatum*-induced specific VOCs, including methyl 2-methylbutyrate, butyl caprylate, and *n*-tetradecane, individually or together, suggesting that it was the mixed blends, but not one specific VOCs, that served as olfactory cues for the host orientation and oviposition selection of YPM females to PDA. This is consistent with the evidence that changes in the overall composition and relative ratios of the host plant VOCs make plants co-infested by *Nilaparvata lugens* (Stål) and *Chilo suppressalis* (Walker) unattractive to *Anagrus nilaparvatae* females (Hu et al., 2020). *One* possible explanation is that, compared with individual components, the quantitative as well as qualitative differences in the blend of plant VOCs have a significant effect on herbivorous insects' behaviors (Bruce and Pickett, 2011). Collectively, these *P. digitatum* induced specific VOCs functioned together as olfactory cues for the interactions between YPM females and apples.

In summary, a battery of experiments were carried out to gain further insight into the hypothetical roles of the plant-associated microbes (*P. digitatum*) in mediating the host plant location and oviposition selection of YPMs *via* host plant VOCs. Current study found that YPM females preferred to *P. digitatum*-infected apples for oviposition in the oviposition behavioral experiments. Odors from *P. digitatum*-infected apples were responsible for the attractiveness of YPM females as demonstrated in the four-arm olfactometer assay. Furthermore, three of five *P. digitatum*-induced specific VOCs, including methyl 2-methylbutyrate, butyl caprylate, and *n*-tetradecane, might serve as key olfactory cues for YPM females to *P. digitatum*-infected apples *via* Y-tube olfactometer assay. These findings shed light on the underlying mechanisms of the attraction of YPMs by *P. digitatum*-infected apples, and might form the basis for the development of attractant formulations for field trapping YPM moths. In the future, field experiments will be carried out to prove the semiochemical roles of individuals as well as blends of *P. digitatum*-infected apple volatiles in attracting YPMs.

Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

YLD and HGG conceived the idea, designed the experiments, and directed the implementation. PPA and SZM performed the experiments. SZM, PPA, HGG, and YLD conducted statistical analyses. MZZ contributed to the conception and design of the study. ZY prepared the materials and lab facilities necessary for this work. HGG and YLD wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1119717/full#supplementary-material

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Bamboo charcoal mediated plant secondary metabolites biosynthesis in tomato against South American tomato pinworm (*Tuta absoluta*)

Limin Chen[1,2,3†], Xiaowei Li[1†], Jinchao Wang[1], Tingting Chen[1], Jinming Zhang[1], Qianggen Zhu[4], Jun Huang[1], Zhijun Zhang[1], Muhammad Hafeez[1], Shuxing Zhou[1], Xiaoyun Ren[1], Wanying Dong[1], Aiwu Jin[4], Youming Hou[2]* and Yaobin Lu[1]*

[1]State Key Laboratory for Managing Biotic and Chemical Threats to Quality and Safety of Agro-Products, Key Laboratory of Biotechnology in Plant Protection, Ministry of Agriculture and Rural Affairs, Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China, [2]State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, Key Lab of Biopesticide and Chemical Biology, Ministry of Education and Fujian Province Key Laboratory of Insect Ecology, College of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou, Fujian, China, [3]Lishui Academy of Agricultural and Forestry Sciences, Lishui, Zhejiang, China, [4]College of Ecology, Lishui University, Lishui, Zhejiang, China

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EDITED BY
Wen Xie,
Insititute of Vegetables and Flowers (CAAS), China

REVIEWED BY
Yibo Zhang,
Institute of Plant Protection (CAAS), China
Yong Liu,
Hunan Academy of Agricultural Sciences (CAAS), China

*CORRESPONDENCE
Yaobin Lu
✉ luybcn@163.com
Youming Hou
✉ ymhou@fafu.edu.cn

†These authors have contributed equally to this work

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Introduction: The south American tomato pinworm (Tuta absoluta), an invasive insect pest species, has established itself in more than 33 Chinese prefectures, where it is mainly damaging tomato crops. Immediate efforts have been initiated to find strategies to control this pest. Studies have shown that biochar (BC) amendment to soil can enhance plant growth and resistance to herbivory.

Methods: First, we quantified the morphological performance of tomato plants grown in different coconut bran and bamboo charcoal (v/v) combinations and selected the most beneficial one. Then we checked the *T. absoluta* survival on the tomato plants grown in the selected bamboo charcoal combination. Finally, we studied the leaf metabolite accumulation and gene expression changes in tomato plants after growing in the selected bamboo charcoal combination.

Results: We found that the 30:1 ratio of bamboo charcoal and coconut bran is the most beneficial to tomato growth as its amendment to soil increased tomato plant height, stem thickness, and chlorophyll content, whereas, the *T. absoluta* survival decreased. The metabolome profiles of BC tomato leaves showed an increased accumulation of flavonoids, terpenoids, and phenolic acids compared to CK. Transcriptome sequencing resulted in the identification of 244 differentially expressed genes. Most of the upregulated genes were associated with stress-related hub proteins, flavonoid biosynthesis, MAPK and phytohormone signaling, and terpenoid biosynthesis. Additionally, the expression of many genes related to signaling and defense was changed in response to the bamboo charcoal amendment.

Discussion: We conclude that bamboo charcoal induces biosynthesis of flavonoids, terpenoids, and phenolic acids, which improve plant growth and tolerance against *T. absoluta*, thus reducing the survival of destructive pests.

KEYWORDS

flavonoids, terpenoids, resistance to insect-pest, South American tomato pinworm, phytohormone signaling, LSU hub genes

1. Introduction

Tuta absoluta (South American tomato pinworm) was a newly invaded insect pest species in Ili, Xinjiang, China in 2017, where it caused significant damage to tomato crops (Zhang et al., 2020a,b). This pest originated in Peru, South America and severely damaged tomato crops in the 1950s. Now it has spread to more than 100 countries in South America, Europe, Africa, and Asia

(Campos et al., 2017; Biondi et al., 2018; Mansour et al., 2018; Han et al., 2019a). Since China is the largest producer of tomato in the world (Ghimire et al., 2017), the rapid spread of *T. absoluta via* fruit and seedling is threatening the Chinese tomato industry. Additionally, the fact that it can also feed on other host plants is alarming to local crop production. A recent survey reported that *T. absoluta* was found in 269 sites in 33 prefectures and the infestation percentage ranged from 78.1 to 100%, which is further alarming and calls for strategies to control this pest.

Among the major control strategies adopted in other countries, chemical control (use of organophosphates, pyrethroids, abamectin, and other broad-range chemicals) remains the principal option to control this invasive insect pest (Desneux et al., 2022). However, this strategy causes severe damage to the environment as well as public health. Furthermore, there are reports on the development of resistance due to short generation time and high biotic potential (Guedes et al., 2019). On the contrary, the biocontrol strategy of using hemipteran mirids has been found to be the most promising due to the ease of their establishment in tomato fields and the high predation rate (Silva et al., 2016). Moreover, nearly 15 arthropod species have been reported to prey on *T. absoluta* (Zappala et al., 2013). However, such biocontrol strategies require rearing of the biocontrol agents on different scales, controlled testing on a domestic scale, and official registration (Yazdanpanah et al., 2021). Among the other strategies, the use of fungi-derived natural products and host plant resistance are suitable from the environment as well as public safety perspectives. Some studies have also reported the adaption of combined approaches such as host plant resistance together with selective insecticides and biocontrol agents (Wangari Nderitu et al., 2020).

One of the non-chemical alternative methods is the use of products derived from agricultural and other organic wastes such as biochar. Biochar amendment to soil has shown pathogen-suppressive effects in a wide range of plant species against plant-pathogenic bacteria, fungi, oomycetes, and nematodes (Poveda et al., 2021). Moreover, the use of biochar amendment can improve plant growth and resistance to herbivory by changing jasmonic acid (JA) levels (Waqas et al., 2018). Studies on tomatoes have shown that biochar application from different feedstocks enhanced plant growth and resistance against *Meloidogyne incognita* (Arshad et al., 2020, 2021). Biochar application can also reduce the reproductive potential of the English grain aphid by activating the expression of defense-related genes in wheat (Chen et al., 2019). Bamboo charcoal is recognized as a regenerative biochar due to its cost-effectiveness and environment friendliness (Kawakami et al., 2005). It has the potential to improve plant growth by improving soil properties, chlorophyll contents, photosynthesis rate, and biomass (Hua et al., 2012). Moreover, its by-products have shown activities against insect pests such as *Tetrenchus cinnabarinus*, *Emposasca flavescens*, and *Aphis medicagini* (Wang et al., 2015). Thus, these reports indicate that the use of bamboo charcoal can improve plant growth and may provide/enhance tolerance against invading insect pests. Based on these reports, we hypothesize that the addition of bamboo charcoal to the soil (or growing substrate) may improve the growth of tomato plants and induce metabolic responses and gene expression that may increase resistance to *T. absoluta* infection. Indeed, various abiotic factors could be manipulated to alter plant phenotypic traits, which could in turn trigger bottom-up effects on insect herbivores and even organisms from higher trophic levels (Han et al., 2022). Much evidence has been obtained for *T. absoluta* management *via* bottom-up effects (Han et al., 2019b).

The use of transcriptome sequencing and metabolome profiling can help us identify the key metabolites and genes that are accumulated or expressed, respectively, in tomato plants grown in soil amended with bamboo charcoal. A similar strategy has helped researchers identify genes and associated pathways such as protein processing, plant-pathogen interaction, photosynthesis, and signaling in response to different biotic stresses e.g., in pepper against *Fusarium wild* (Zhu et al., 2021), in oat against oat-stem-rust (Li et al., 2022), and in strawberry fruits against *Botrytis cinerea* (De Tender et al., 2021). In this study, we used a combined metabolome profiling and transcriptome sequencing approach to understand the key changes in the accumulated metabolites and respective changes in gene expression in leaves of tomato plants grown in coconut bran amended with bamboo charcoal. We also studied if bamboo charcoal amendment effects the survival of *T. absoluta* on tomato plants. First, we quantified the morphological performance of tomato plants grown in different coconut bran and bamboo charcoal (v/v) combinations and selected the most beneficial one. Then we checked the *T. absoluta* survival on the tomato plants grown in the selected bamboo charcoal combination. Finally, we studied the leaf metabolite accumulation and gene expression changes in tomato plants after growing in the selected bamboo charcoal combination.

2. Materials and methods

2.1. Plant material and growth in bamboo biochar

The tomato (*Solanum lycopersicum* L.) variety "Moneymaker" was used as the plant material. The seeds were obtained from Zhejiang Academy of Agricultural Sciences, China. Seeds were surface sterilized and sown in square pots (7 cm wide and 9 cm deep) filled with sterilized coconut bran in an insect-free greenhouse at the experimental station of the Zhejiang Academy of Agricultural Sciences. The day/night temperatures and relative humidity were 24 ± 1/20 ± 1°C and 60 ± 5%. The experimental plants were not exposed to any type of pesticides or herbicides. The experimental plants (treated and CK) were supplemented with water-soluble macro-element fertilizer (OMEX, 18-18-18 NPK) at 2 g per pot to promote plant growth. When the seedlings reached two-leaf stage, they were transferred into different combinations of the sterilized coconut bran and bamboo charcoal v/v mixtures i.e., 10:1 (BC1), 30:1 (BC2), and 50:1 (BC3), whereas, 100% coconut bran was used as control (CK). Each treatment was repeated three times with fifteen plants per repetition (35 plants per treatment). The pots were watered every 2 days. The physiological parameters i.e., plant height and stem diameter were measured on the 10th and 20th day after seedling transplanting. For each trait measurement, eleven plants were randomly selected from each treatment. The means of three replicates were compared by the least significant difference test in Microsoft Excel® 2019 (www.microsoft.com). The most useful mixture (v/v) of coconut bran and bamboo charcoal was selected.

2.2. Insect feeding of BC-grown tomato plants and survivorship of *T. absoluta*

The South American tomato pinworm (*T. absoluta*) population was collected from Yili, Xinjiang, China in 2018, and was continuously raised in an artificial climate chamber with temperature, relative humidity, and light/dark period of 25 ± 1°C, 60 ± 5%, and 16/8 h, respectively. Tomato plants reaching five-leaf stage were used for experiments. Ten plants (six repeats for BC and CK each) were put in 50 × 50 × 50 cm^3 nylon gauze insect cages and the newly emerged *T. absoluta* larvae were placed individually on each tomato plant for 12 days. In total, 120 newly emerged *T. absoluta* larvae were randomly allocated to the two treatments (BC and CK). The individual larvae on the tomato leaf were checked daily for development, and the pupated larvae were recorded as alive. Survival rates were recorded and statistically tested ($p < 0.05$) by using SPSS (version 26). The probability of survival was determined by using a Log-Rank test and the survival distributions of the *T. absoluta* on BC and CK plants were compared (GraphPad Prism 9).

2.3. Metabolome analysis

2.3.1. Sample preparation and extraction

Healthy tomato leaf samples (in triplicate) were collected from seedlings transplanted after 20 days. The sampled leaves were washed with distilled water, immediately frozen in liquid nitrogen, and stored in a −80°C freezer. The leaf samples were freeze-dried by vacuum freeze-dryer (Scientz-100F) and crushed using a mixer mill (MM 400, Retsch) with the help of zirconia beads for 1.5 min at 30 Hz. The lyophilized powder (50 mg) was then dissolved in 1.2 mL of 70% methanol solution, followed by vertexing for 30 s. The vertexing was done six times every 30 min. The mixture was then centrifuged at 12,000 rpm for 3 min, the extracts were filtered through a 0.22 μm microfilter (SCAA 104, ANPEL, Shanghai, China), and analyzed in UPLC-MS/MS.

2.3.2. UPLC conditions and ESI-Q TRAP-MS/MS

The extracts were analyzed using a UPLC-ESI-MS/MS system (UPLC, SHIMADZU Nexera X2, https://www.shimadzu.com.cn/; MS, Applied Biosystems 4,500 Q TRAP, Thermofisher, China). The column was Agilent SB-C18 (1.8 μm, 2.1 mm ×100 mm); The mobile phase consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. For the sample measurements, we used a gradient program with the starting conditions of 95% A, 5% B. Within 9 min, a linear gradient to 5% A, 95% B was programmed, and a composition of 5% A, 95% B was kept for 1 min. Afterwards, a composition of 95% A, 5.0% B was adjusted within 1.1 min and kept for 2.9 min. The flow velocity was set as 0.35 mL per minute; the column oven was set to 40°C; the injection volume was 4 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

The ESI source operation parameters were as follows: source temperature 550°C; ion spray voltage (IS) 5,500 V (positive ion mode)/-4,500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), and the curtain gas (CUR) were set at 50, 60, and 25 psi, respectively; the collision-activated dissociation (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to medium. DP (declustering potential) and CE (collision energy) for individual MRM transitions was done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

2.3.3. Bioinformatics analyses of metabolome data

Principal Component Analysis (PCA) was computed by using the "prcomp" function in R (www.r-project.org). Pearson correlation coefficients (PCC) between samples were calculated by the "cor" function in R and presented as heatmaps.

The metabolites were considered differentially accumulated if the variable importance in projection (VIP) was ≥ 1 and the absolute Log_2 FC was ($|\text{Log}_2\ \text{FC}| \geq 1.0$). The VIP values were extracted from OPLS-DA results, which were generated using the R package MetaboAnalystR. The data was log-transformed and mean centered before OPLS-DA. In order to avoid overfitting, a permutation test (200 permutations) was performed. The identified metabolites were annotated using the KEGG Compound database (http://www.kegg.jp/kegg/compound/), and annotated metabolites were then mapped to the KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html). Pathways to which the differentially accumulated metabolites (DAMs) were mapped were fed into MSEA (metabolite sets enrichment analysis), and their significance was determined by the hypergeometric test's *p*-values.

2.4. Transcriptome sequencing

2.4.1. RNA extraction, library preparation, and sequencing

Total RNAs were extracted from triplicate leaf samples by using Spin Column Plant total RNA purification Kit (Sangon Biotech, China). RNA integrity and purity were tested by agarose gel electrophoresis and NanoPhotometer, respectively. RNA concentration was measured using spectrophotometer and Qubit 2.0 Fluorometer. PolyA tail enrichment of RNAs was done through Oligo (dT) magnetic beads to obtain mRNAs. The mRNAs were fragmented by using fragmentation buffer, and were used to synthesize cDNA using a kit (QuantiTech Reverse Transcription Kit, Qiagen). The cDNAs were purified by AMPure XP beads, A-tailed, and ligated with sequencing adapters. The fragment size selection was done by using AMPure XP beads, and cDNA libraries were obtained, quantified, and their insert size was detected by 2,100 bioanalyzer (Agilent Technologies, California, United States). q-PCR was used for quantification of an effective library concentration i.e., >2 nM. After finding out the effective library concentration, libraries were pooled, and sequenced on the Illumina HiSeq platform.

2.4.2. Bioinformatic analyses

For analyzing the sequencing data, first, we filtered the data and obtained high-quality reads followed by calculating the sequencing error rate and GC content distribution (Petrussa et al., 2013). Next, the transcriptome sequencing data was compared with the reference genome (Sola-num_lycopersicum.SL3.0) using HISAT2 (Wang et al., 2020). Then, BLAST was used to compare the transcript sequences with KEGG (Mukhtar et al., 2011) database. The transcript expression was computed as Fragments Per Kilobase of transcript per Million fragments mapped (FPKM). Finally, PCC and PCA were computed in R.

Differential gene expression between CK and the treatment group was computed as reported earlier (Liang et al., 2022) by using DESeq2 (Koch et al., 2016). The Benjamini Hochberg method was used to perform multiple hypothesis test corrections on the probability (*P*-value) to obtain false discovery rate (FDR). The screening criteria for the differentially expressed genes (DEGs) were $|\log_2 \text{Fold Change}| \geq 1$ and $\text{FDR} < 0.05$. Venn diagrams were prepared in InteractiVenn (Farhangi-Abriz and Torabian, 2018). Pathway annotation of the DEGs was done in KEGG (https://www.genome.jp/kegg) (Mukhtar et al., 2011). The enrichment of DEGs in different KEGG pathways was done as reported earlier (Petrussa et al., 2013).

To validate the RNA sequencing-based expression, 15 tomato genes were selected for qRT-PCR analysis using the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA) and the iTaq Universal SYBR Green Supermix 50 ml (BIO-RAD, Hercules, CA, USA). cDNAs produced during the RNA sequencing stage were used and four biological and three technical replicates were included. The qRT-PCR was based on specific primer pairs (Supplementary Table 1) designed in Primer 3.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Ding et al., 2019). *TUB* gene was used as the reference (Supplementary Table 1).

3. Results

3.1. Morphological performance of tomato seedlings under different bamboo charcoal (v/v) ratios

Plant height of BC1 (coconut bran and bamboo charcoal v/v 10:1), BC2 (coconut bran and bamboo charcoal v/v 30:1), and BC3 (coconut bran and bamboo charcoal v/v 50:1) treatments increased by 11.2, 16.59, and 24.35%, respectively, compared to CK 10 days after the seedlings transplantation. Whereas, after 20 days of transplanting, the plant height of tomato seedlings significantly increased in BC2, but no significant difference was found in BC1 and BC3 compared to CK (Figures 1A, Bi, Supplementary Table 2A). These results suggest that BC2 is suitable combination for tomato seedling growth. After seedlings were transplanted for 10 days, the stem thickness increased in BC3, however, in the case of BC1 and BC2, there was no significant increase as compared to CK. While, stem thickness of the seedlings transplanted after 20 days increased significantly in BC1 and BC2 but the increase was not significant in BC3 (5.06 cm) compared to CK (4.72 cm) (Figure 1Bii, Supplementary Table 2A). These observations further confirm the plant height results that BC2 is the most suitable combination of coconut bran and bamboo charcoal. Finally, the chlorophyll content was significantly different in BC1 as compared to CK in seedlings transplanted after 10 days, whereas the chlorophyll content of BC1 was obviously lower than that of other treatments in seedlings transplanted after 20 days (Figure 1Biii, Supplementary Table 2A). Thus, the plant height, stem thickness, and chlorophyll contents indicate that BC2 is the best mixture that supports tomato seedling growth. Also, these observations indicate that seedlings transplanted after 20 days are a better choice to understand the effect of BC on the growth and chlorophyll content as compared to CK (Figure 1).

3.2. Feeding of *T. absoluta* on tomato seedlings' leaves grown in BC

The *T. absoluta* were fed on the tomato seedling leaves grown in BC2 (thereafter BC) and CK. The probability of the survival of *T. absoluta* significantly decreased with increase in days as compared to CK (Figure 2A). Similarly, the survival rate of the *T. absoluta* was significantly lower in BC as compared to CK (Figure 2B). These observations indicate that BC significantly improves the tolerance of tomato seedlings against *T. absoluta*.

3.3. Metabolomic response of tomato seedlings grown in bamboo charcoal

The metabolome profiles of BC and CK leaves could detect 919 metabolites that were classified as alkaloids, amino acids and derivatives, flavonoids, lignans and coumarins, lipids, nucleotides and derivatives, organic acids, phenolic acids, steroids, and terpenoids (Figure 3A). Principal component analysis showed that BC and CK replicates tended to group together (Figure 3B), whereas PCC was >0.93 (Figure 3C). Both the PCA and PCC analyses indicate that the sampling was reliable. Almost all compound classes were accumulated in higher quantities in BC as compared to CK (Figure 3D).

3.4. Differential metabolome profiles of tomato leaves grown in CK and BC

Based on the screening criteria i.e.., $\text{VIP} \geq 1$ and $|\text{Log}_2 \text{ FC}| \geq 1.0$, 39 metabolites were differentially accumulated between BC and CK. These metabolites were classified as flavonoids, amino acids and derivatives, alkaloids, lipids, nucleotides and derivatives, terpenoids, and steroids (Figure 4A). Six metabolites (Dihydrokaempferol-3-O-glucoside, Querce-tin-3-O-glucosyl(1→ 4)rhamnoside-7-O-rutinoside, Querce-tin-3-O-(6"-O-p-coumaroyl)sophoroside-7-O-rhamnoside, p-Coumaroyleuscaphic acid, Dihy-drokaempferol-7-O-glucoside, and Neotigogenin-glucose-glucose-glucose-glucoside) were exclusively detected in BC. In addition to these six compounds, four other compounds belonging to phenolic acid (1) and flavonoids (3) were highly up-accumulated in BC as compared to CK (Figure 4B). Most of the other up-accumulated metabolites in BC were classified as flavonoids,

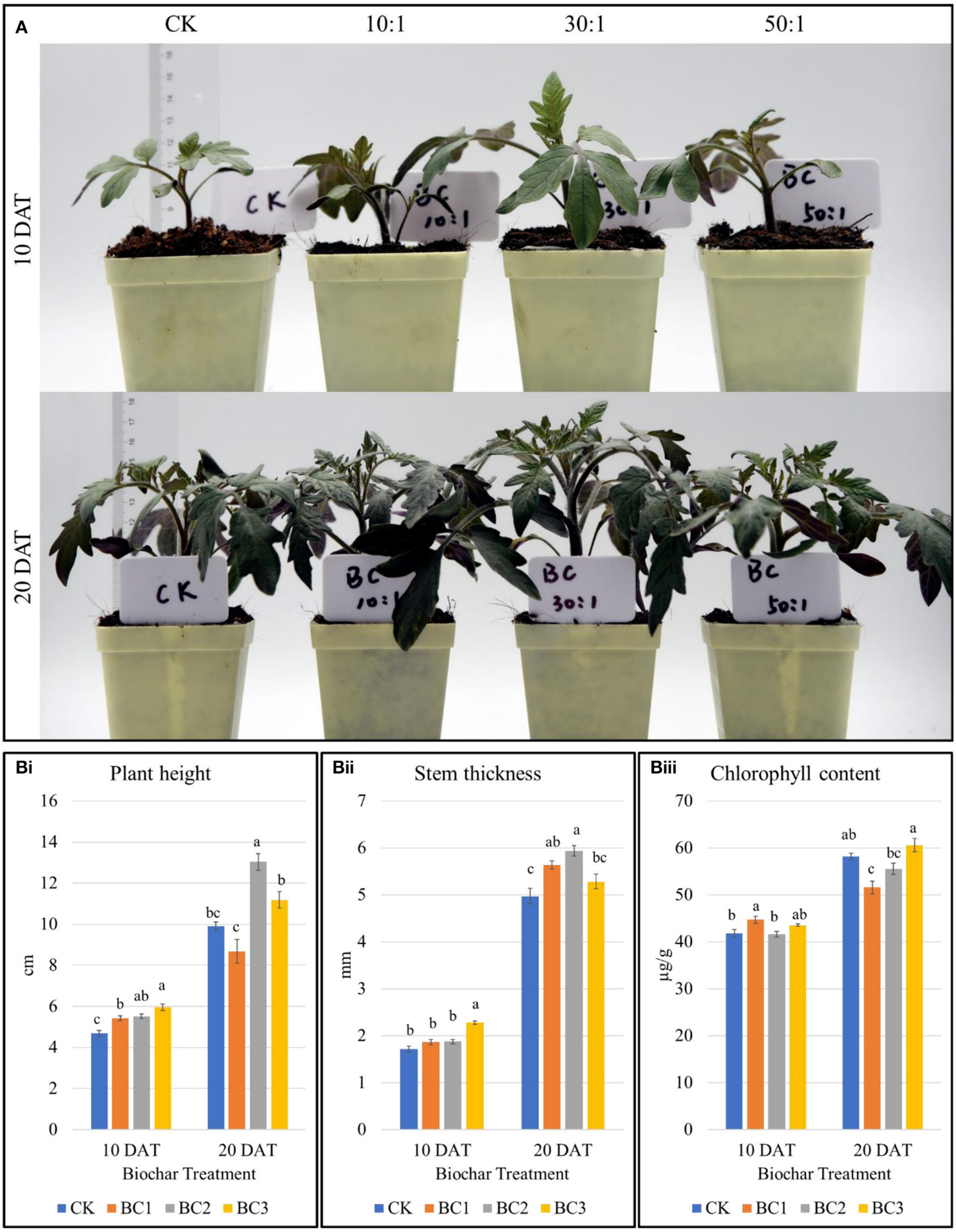


FIGURE 1
(A) Phenotypes of the tomato plants grown in three different coconut bran and bamboo charcoal combinations. **(Bi)** Plant height **(Bii)**, stem thickness, and **(Biii)** chlorophyll content in tomato leaves grown in BC1 (10:1), BC2 (30:1), and BC3 (50:1). The bars show mean ± SEM ($n = 11$). Different letter in figure b indicates that differences in treatments are significant ($p < 0.05$).

terpenoids, phenolic acids, organic acids, and steroidal saponins (Supplementary Table 3). These observations indicate that BC leaves have higher flavonoid, terpenoid, organic acid, and phenolic acid contents than CK, therefore, growing tomato in 30:1 v/v coconut bran and bamboo charcoal induces their increased biosynthesis.

On the contrary, the top-10 most accumulated compounds in CK were lipids (1), flavonoids (2), amino acids and derivatives (4), nucleotides and derivatives (2), and alkaloids (1) (Figure 4B). Taken together, higher accumulation of flavonoids, organic acids (3-Methyl-2-oxopentanoic acid), phenolic acids, steroids (Neotigogenin-

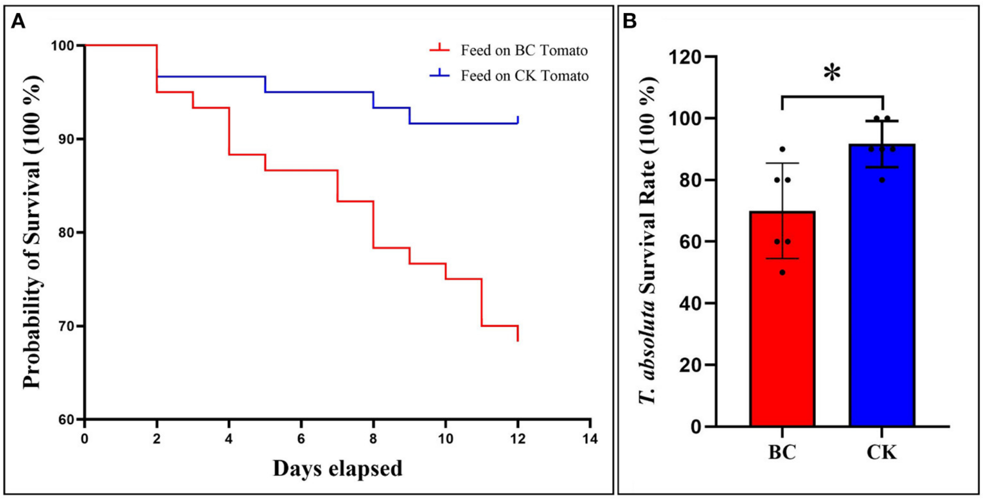
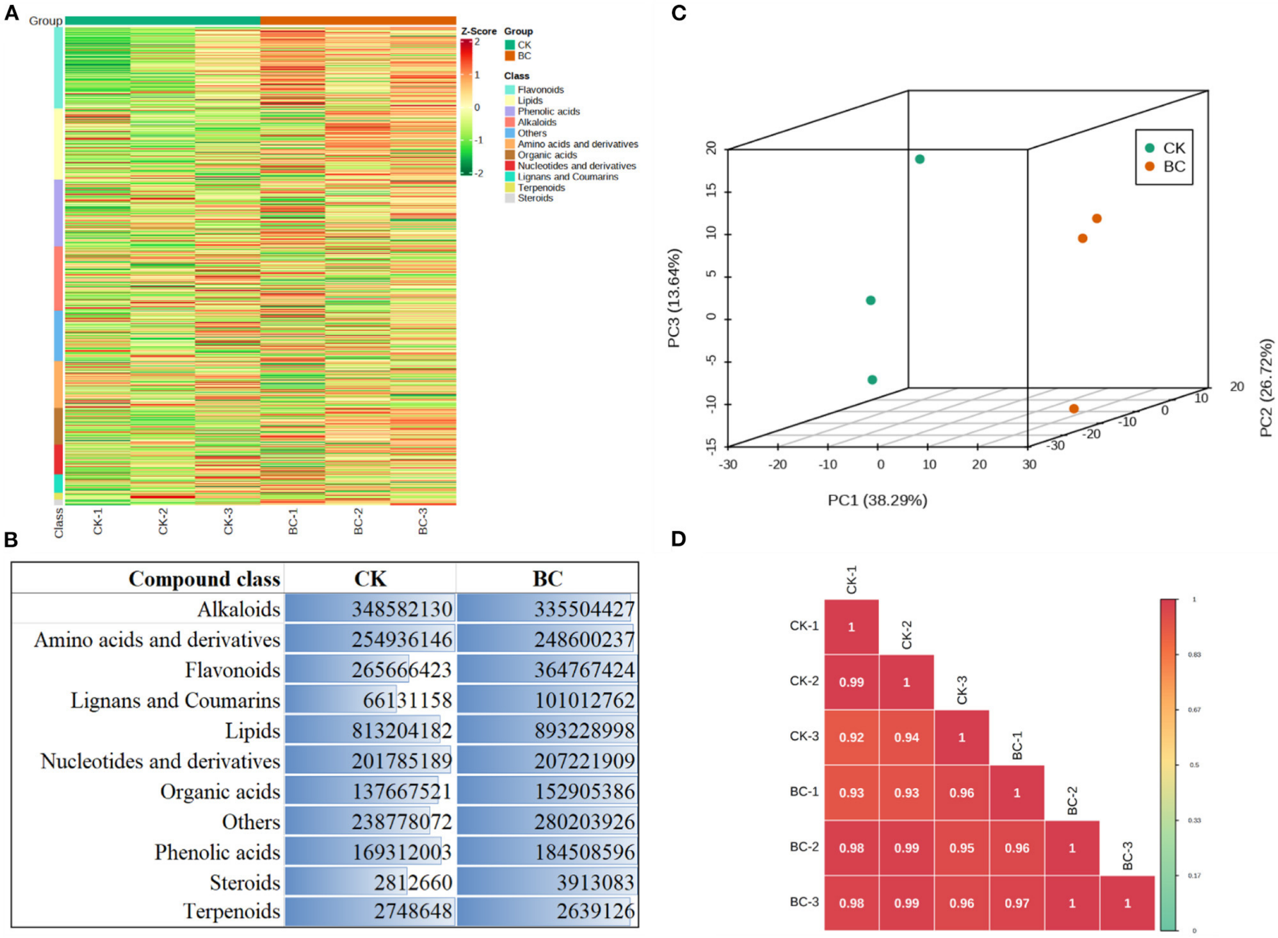


FIGURE 2
(A) Probability of survival of *T. absoluta*. **(B)** *T. absoluta* survival rate fed on BC and CK. CK (100% coconut bran), BC (30:1 v/v coconut bran and bamboo charcoal). The bars show mean ± SEM ($n = 6$). *Indicates significant differences between the CK and BC ($p < 0.05$).



Compound class	CK	BC
Alkaloids	348582130	335504427
Amino acids and derivatives	254936146	248600237
Flavonoids	265666423	364767424
Lignans and Coumarins	66131158	101012762
Lipids	813204182	893228998
Nucleotides and derivatives	201785189	207221909
Organic acids	137667521	152905386
Others	238778072	280203926
Phenolic acids	169312003	184508596
Steroids	2812660	3913083
Terpenoids	2748648	2639126

FIGURE 3
Metabolome analysis of BC (30:1 v/v coconut bran and bamboo charcoal) and CK (100% coconut bran) leaves. **(A)** Heatmap of the relative intensities of the detected metabolites in CK vs. BC, **(B)** Sum of accumulated contents of metabolite classes in CK and BC, **(C)** Principal component analysis, and **(D)** Pearson's Correlation Coefficient based on the relative metabolite intensities in CK vs. BC.

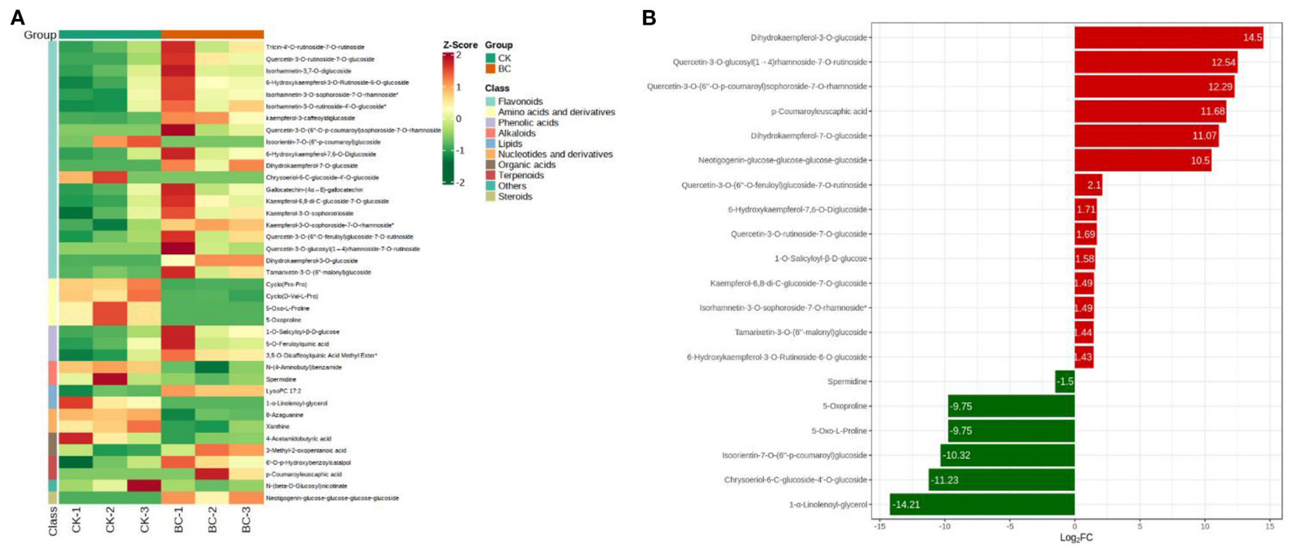


FIGURE 4
Differential metabolome profile of tomato seedlings grown in CK (100% coconut bran) and BC (30:1 v/v coconut bran and bamboo charcoal). **(A)** Heatmap of the z-scores and **(B)** top-10 up- and down accumulated metabolites in CK vs. BC.

glucose-glucose-glucose-glucoside), and terpenoids in BC can be related with the decreased survival rate of *T. absoluta*.

3.5. Transcriptome response of tomato seedlings grown in bamboo charcoal

3.5.1. Summary of transcriptome sequencing

Sequencing of six tomato leaf cDNA libraries resulted in 43.98 to 53.47 million raw reads. After filtering, 42.08–52.15 million clear reads (41.38 Gb clean data) were obtained. The Q20%, Q30%, GC content (%), and error rate (%) were 98.1, 94.227, 42.901, and 0.025%, respectively (Supplementary Table 4). Gene expression was computed as FPKM (Supplementary Figure 1A). The FPKM-based PCA analysis showed that PC1 and PC2 explained 28.41 and 23.98% variation, respectively (Supplementary Figure 1B). The PCC between the replicates of the same treatment and/or control was > 0.95, indicating the reliability of the sampling (Supplementary Figure 1C). A total of 26,046 transcripts were expressed, which could be annotated in GO, KEGG, KOG, NR, Pfam, and SwissProt. The datasets of the transcriptomes from our study were uploaded to NCBI Sequence Read Archive with accession number PRJNA898145.

3.5.2. Differential gene expression profiles of tomato leaves (CK vs. BC)

A total of 224 transcripts were differentially expressed between CK and BC; 144 were downregulated, while 80 were upregulated in BC. These transcripts were enriched in environmental information processing (ABC transporters, MAPK signaling, and plant hormone signal transduction), metabolism, and organismal systems (circadian rhythm and plant-pathogen interaction) (Figures 5A, B). According to GO classification, the highest number of DEGs were related to cellular components (cellular anatomical entity), followed by molecular processes (cellular processes and metabolic processes), and molecular function (catalytic activity and binding) (Figure 5C). The qRT-PCR analysis of 15 randomly selected genes indicated similar transcript levels as of RNA-seq results (Supplementary Figure 2).

3.5.2.1. Highly up and downregulated genes in BC-grown tomato seedlings

The most upregulated gene in BC was RESPONSE TO LOW SULFUR 2 (LSU2)/EN-HANCED DE-ETIOLATION 6 (*Solyc03g096780.1*), followed by LSU3 (*Solyc03g096770.1*), L-ornithine N5-acetyltransferase (*Solyc00g272810.1*), acyl-lipid Delta6-acetylenase/acyl-lipid (9-3)-desaturase (*Solyc08g063090.2*), and Probable serine/threonine-protein kinase PBL2 (*novel.1843*). Whereas, multicystatin (*novel.2673*), wound-induced proteinase inhibitor (*Solyc09g089500.3, Solyc09g089540.3,* and *Solyc09g083445.1*), proteinase inhibitor I-B-like (*Solyc09g089530.3*), leucyl aminopeptidase (*Solyc12g010030.2* and *novel.2698*), serine protease inhibitor 5-like (*Solyc03g098760.2*), were downregulated in BC as compared to CK (Supplementary Table 4).

3.5.2.2. Changes in the expressions of MAPK and plant hormone signaling-related genes

Three LRR receptor-like serine/threonine-protein kinase ERECTA (*Solyc03g112580.3, Solyc05g015150.3,* and *Solyc03g007050.3*), and abscisic acid receptor PYR/PYL (*Solyc06g050500.2*) enriched in MAPK signaling pathway—plant were upregulated in BC as compared to CK. Whereas, a protein phosphatase 2C (*Solyc03g096670.3*), a WRKY22 (*Solyc03g007380.2*), and a TF MYC2 (*Solyc10g009270.3*) were downregulated in BC as compared to CK. Other than MAPK signaling, we also observed that phytohormone signaling-related genes were also differentially expressed. Of these, protein brassinosteroid insensitive 1 (BRI1, *Solyc00g110870.3*) and a DELLA protein (*Solyc07g065270.1*) were up-regulated in BC as compared to CK, whereas, others including xyloglu-can:xyloglucosyl transferase TCH4 and two gibberellin receptor GID1 were downregulated in BC (Supplementary Table 4).

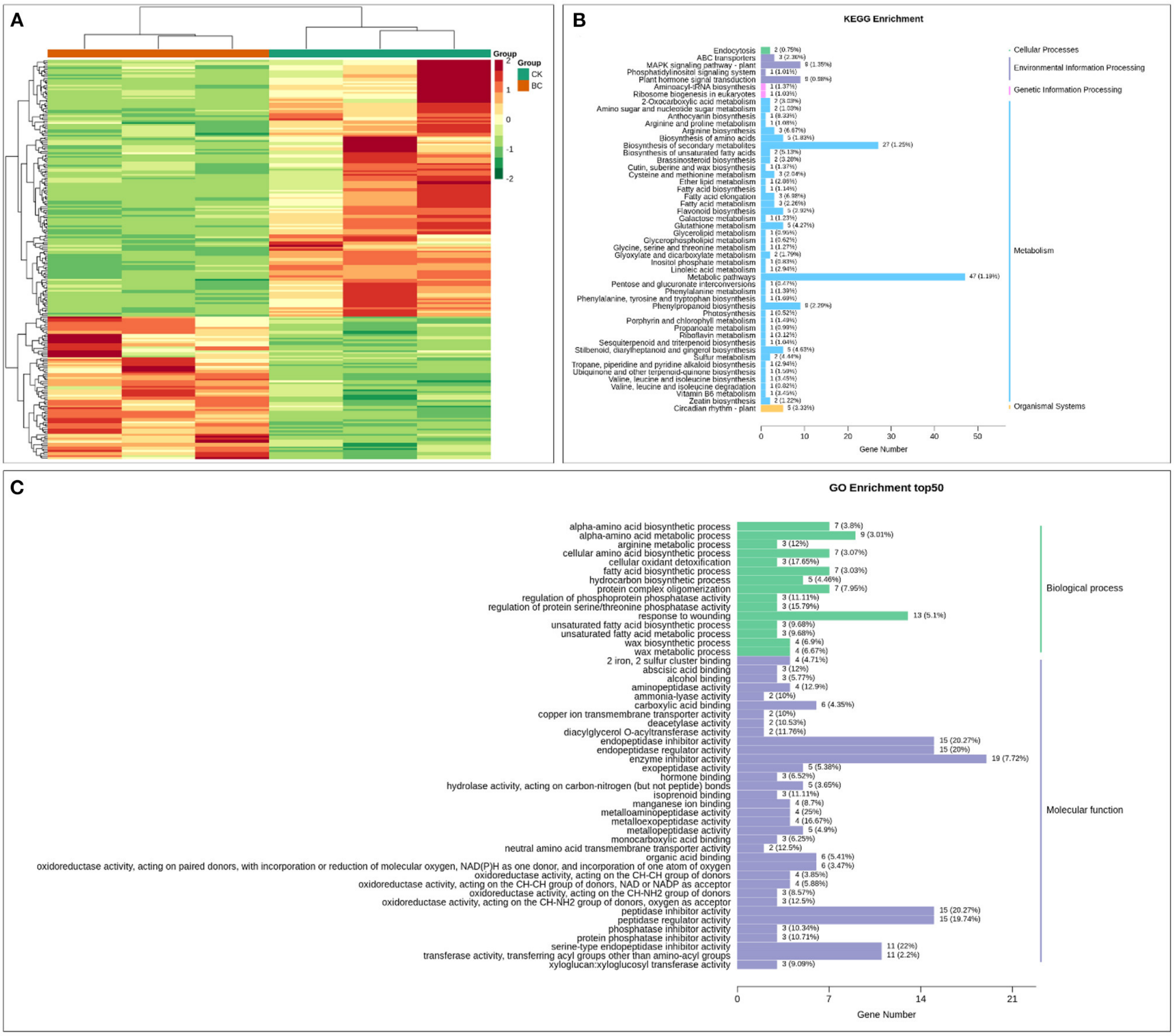


FIGURE 5
(A) Heatmap of FPKM values of the differentially expressed genes in CK vs. BC. Enrichment of the differentially expressed genes in CK vs. BC in **(B)** KEGG and **(C)** GO pathways.

These observations indicate that gibberellin, ABA, brassinosteroid, and salicylic acid signaling play roles in better performance of the BC-grown tomato seedlings as compared to CK.

By removing the $\log_2$ foldchange screening criteria, overall, there were 705 genes associated with phytohormone signaling. All these showed variable gene expressions. However, interesting observations were that the brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1) and most of the protein BRI1 were upregulated in BC as compared to CK. Similarly, the MAPKKK17/18 and MAPKK ANP1 transcripts were upregulated in BC as compared to CK (Supplementary Table 5).

3.5.2.3. Changes in the expressions of flavonoid, terpenoid, sugar-related genes

Three genes i.e., nerolidol synthase (*Solyc10g005400.3*), anthocyanidin 3-O-glucoside 2"'-O-xylosyltransferase (*Solyc04g079050.2*), and tropinone reductase 1 (*Solyc06g083470.3*) were upregulated in BC as compared to CK, whereas several other genes enriched in phenylpropanoid biosynthesis, flavonoid biosynthesis, and stilbenoid, diarylheptanoid, and gingerol biosynthesis were downregulated in BC as compared to CK. To further find related genes, we searched the GO terms with annotation as "terpenoids." This resulted in the identification of five genes; three of which were upregulated in BC as compared to CK including cytochrome P450 family 76 subfamily A (*Solyc09g098030.3*), solute carrier family 15 (pep-tide/histidine transporter), member 44624 (*Solyc03g113250.3*), and a typhasterol/6-deoxotyphasterol 2alpha-hydroxylase (*Solyc00g170200.1*). Whereas, two genes were downregulated in BC as compared to CK; cis-zeatin O-glucosyltransferase (*Solyc11g007490.2*) and strigolactone esterase (*Solyc00g170200.1*). The GO terms associated with "polysaccharides" included the basic endochitinase B, beta-amylase, and subtilisin-like protease SBT1.9. These transcripts were downregulated in BC as compared to CK, whereas, xyloglucan:xyloglucosyl transferases, pectinesterase, and a Subtilisin-like protease SBT3 were upregulated in BC as compared to CK (Supplementary Table 4).

We also searched for genes involved in flavonoid biosynthesis regardless of DEG filtering criteria and found 28 transcripts related to flavonoid biosynthesis e.g., chalcone synthase, flavonoid 3′-monooxygenase, shikimate O-hroxycinnamoyltransferase, flavanone 4-reductase, and flavonoid 3′,5′-hydroxylase had higher expressions in BC as compared to CK. These changes are consistent with the observed higher flavonoid accumulation in BC as compared to CK. Moreover, 88 genes related to terpenoid biosynthesis or terpenoid backbone biosynthesis showed higher expressions in BC as compared to CK. Notably, we observed higher expressions of genes annotated as 8-dydroxygeraniol dehydrogenase, (-)-germacrene D synthase, premnaspirodiene oxygenase, (3S,6E)-nerolidol synthase, geranylgeranyl diphosphate synthase, type II, geranylgeranyltransferase type-1 subunit alpha, 4-coumarate-CoA ligase, and squalene monooxygenase in BC as compared to CK. These changes are also consistent with the metabolome profiles of BC and CK. Similarly, 91 genes related to starch and sucrose biosynthesis had higher expressions in BC as compared to CK. Most importantly, we observed the higher expression of starch synthases, trehalose 6-phosphate, endoglucanases, glucan endo-1,3-beta-glucosidases, and trehalose 6-phosphate synthase/phosphatase. These expressions are consistent with the higher accumulation of saccharides in BC as compared to CK (Supplementary Table 5).

Taken together, the transcriptome data confirms that metabolomic profiles are related to flavonoids, terpenoids, and saccharides in BC.

3.5.2.4. Changes in expression of fatty acid and cutin, suberine, and wax-related genes

Twelve DEGs were enriched in fatty acid-related pathways i.e., fatty acid elongation, fatty acid biosynthesis, biosynthesis of unsaturated fatty acids, fatty acid metabolism, and cutin, suberin, and wax biosynthesis. Only four genes associated with these pathways were upregulated. Of these, the 3-ketoacyl-CoA synthase (Solyc05g009270.3), acyl-lipid Delta6-acetylenase/acyl-lipid (9-3)-desaturase (Solyc08g063090.2), solute carrier family 15 (peptide/histidine transporter), member 44624 (Solyc01g096880.3), and the anthocyanin 3-O-glucoside 2-oxylosytransferase were up-regulated in BC as compared to CK (Supplementary Table 4).

Further exploration of genes with fatty acid biosynthesis related annotations showed that 32 transcripts were increasingly expressed in BC as compared to CK. These include 3-oxoacyl-[acyl-carrier protein] reductase, long-chain acyl-CoA synthetase, acetyl-CoA carboxylase, acyl-[acyl-carrier-protein] desaturase, 3-oxoacyl-[acyl-carrier-protein] synthase II, and fat-ty acyl-ACP thioesterase B (Supplementary Table 5).

3.5.2.5. Differential expression of sulfur metabolism, glutathione metabolism, and solute carrier family genes in CK vs. BC

Two genes annotated as adenylyl-sulfate reductase (glutathione) enriched in sulfur metabolism were upregulated. Eight genes annotated as solute carrier family members were differentially ex-pressed. Of these, only solute carrier family 15 members (*Solyc03g113250.3* and *Solyc01g096880.3*) were upregulated in BC, whereas all others including solute carrier family 31, 36, 39, and 50 members were downregulated in BC as compared to CK. Five genes [glutathione S-transferase (GST, *Solyc06g069045.1*) and four leucyl aminopeptidases (*Solyc12g010020.2*, *Solyc12g010025.1*, *novel.2698*, and *Solyc12g010030.2*)] were downregulated in BC as compared to CK. However, by considering genes with $\log_2$ foldchange lower than 1 and higher than -1, we observed that multiple GST transcripts, L-ascorbate peroxidases, glutathione dehydrogenase/transferases, and glucose-6-phosphate 1-dehydrogenases that were associated with glutathione metabolism had higher FPKM values in BC as compared to CK (Supplementary Tables 4, 5).

Regarding the changes in the expression of transcription factors (TFs), of the 26,046 expressed genes, 1,850 were annotated as TFs, which were classified as 91 TF families. Interestingly, 789 TFs showed upregulation; the most upregulated TFs belonged to AP2/ERF, LOB, bHLH, GNAT, SET, SWI, B3, and WRKY families (Supplementary Table 6).

4. Discussion

Biochar amendment in soil/substrate has shown positive effects on plant defense against different pathogens, however, limited data is available for insect pests (De Tender et al., 2021). The spread of *T. absoluta* in mainland China (Zhang et al., 2021) calls for the exploration of possible strategies to reduce tomato crop losses. Several studies have also reported positive effects of bamboo charcoal on plant growth and development. Our results that BC (30:1) caused a significant increase in stem thickness and plant height indicate that the usage of bamboo charcoal in a specific v/v ratio could be beneficial for plant growth and development. This observation is consistent with earlier reports that different types and ratios of biochar have shown variations in their effects on plants (Chrysargyris et al., 2020). Our results are also consistent with the earlier reports that incorporation of bamboo charcoal in growth media increased plant height in tea (Gao et al., 2012), stem diameter in *Sapium sebiferum* (L.) and some temperate broad-leaved trees (Chen et al., 2021), and chlorophyll content in turfgrass (Hua et al., 2012). Thus, we conclude that bamboo charcoal has growth promoting effects on tomato seedlings. In addition, the results that the survival of *T. absoluta* significantly decreased in BC as compared to CK indicate that its addition increases tomato plants' resistance against *T. absoluta*. Previously, soil amendment with biochar has shown to have the ability to increase plant's resistance to herbivory (Waqas et al., 2018) and *M. incognita* infestation (Arshad et al., 2020, 2021). The metabolomic and transcriptomic signatures that are related to such changes in tolerance against *T. absoluta* are discussed below.

Biochar exerts beneficial effects on plant growth and development. Such effects also cause changes in the biosynthesis of plant primary and secondary metabolites. Earlier studies have shown that the application of biochar causes metabolic shift in different plant organs (Sun et al., 2017). Particularly, biochar application caused significant increase in flavonoid, total sugars, and enzyme activities in basil, and resulted in higher plant height, leaf length, leaf number, and yield (Jabborova et al., 2021). Our data also suggest that the use of bamboo charcoal can result in a higher accumulation of flavonoids, phenolic acids, steroids, and organic acids in tomato leaf (Figure 4). Flavonoids (Lattanzio et al., 2000), organic acids (Morgunov et al., 2017), steroids (Janson et al., 2009) have been implicated in both the plant growth and survival as well as defense against insect pests. Therefore, the higher content of these secondary metabolites is possibly related to the decreased *T. absoluata* survival rate. One of the inducible chemical defense responses in plants against invading insect pests and herbivores is the synthesis of a wide range of secondary metabolites including flavonoids, terpenoids, and alkaloids (D'Esposito et al., 2021). Our data also indicated a higher accumulation of flavanonols, flavonols, triterpenes, steroidal saponins, phenolic acids, flavones, monoterpenoids, and phenolic acids (Supplementary Table 3). These observations are consistent with the report that biosynthesis of defensive secondary metabolites increased in a partially resistant cherry tomato in response to *T. absoluta* (D'Esposito et al., 2021). Therefore, from the literature cited and our combined metabolome and transcriptome data, we can conclude that the biosynthesis of these metabolites is a defense response in tomato against *T. absoluta* and growing in bamboo charcoal can enhance the accumulation of these metabolites in tomatoes.

To further understand the changes in total relative intensities of these metabolite classes in BC, we used the transcriptome sequencing approach. The upregulation of nerolidol synthase transcripts in BC is consistent with earlier report that it is an herbivore-inducible terpene synthase gene in maize (Degenhardt and Gershenzon, 2000). Similar to this report, the higher expression level of the nerolidol synthase transcripts in BC as compared to CK might be a reason for the observed reduced *T. absoluta* survival rate (Figure 2). Furthermore, the increased expression of other terpene biosynthesis related genes, such as 8-dydroxygeraniol dehydrogenase, (-)-germacrene D synthase, premnaspirodiene oxygenase, (3S,6E)-nerolidol synthase, geranylgeranyl diphosphate synthase, type II, geranylgeranyltransferase type-1 subunit alpha, 4-coumarate-CoA ligase, and squalene monooxygenase, is also consistent with higher terpenoid accumulation in BC (Supplementary Table 3). Thus, it is understandable that bamboo charcoal induces higher expression of terpenoid biosynthesis related genes that lead toward higher terpenoid accumulation and reduced *T. absoluta* survival on tomato leaves. Similarly, the higher flavonoids content and respective upregulation of flavonoid-biosynthesis genes including chalcone synthase, flavonoid 3'-monooxygenase, shikimate O-hydroxycinnamoyltransferase, flavanone 4-reductase, and flavonoid 3′,5′-hydroxylase (Petrussa et al., 2013), indicate that bamboo charcoal induces higher expression in flavonoids. This higher flavonoid biosynthesis in turn enables tomato plants to tolerate *T. absoluta* as they play defensive roles in other plant species against insect pests (Lattanzio et al., 2000). Moreover, our data also indicated that bamboo charcoal induces higher expression of starch and sucrose biosynthesis, which is consistent with the earlier reports that biochar amendment can improve the total sugar contents in plants (Sun et al., 2017; Jabborova et al., 2021).

The higher expression of LSU transcripts indicates that bamboo charcoal induces stress-related hub proteins. The LSU proteins have been identified as immune-related hubs and play undefined roles against pathogens (Mukhtar et al., 2011). Furthermore, LSU proteins are involved in various protein-protein interactions with the proteins functioning at different molecular levels (Mukhtar et al., 2011). For example, reduced expressions of LSU genes in Arabidopsis resulted in increased disease (*Pseudomonas syringae* infection) susceptibility (Garcia-Molina et al., 2017). Therefore, it is possible that LSU proteins perform similar functions in tomato against *T. absoluta*, and that their expressions can be induced by growing tomato in bamboo charcoal. The upregulation of genes such as L-ornithine N5-acetyltransferase, acyl-lipid delta6-acetylenase, and probable serine/threonine-protein kinase PBL2 in response to bamboo charcoal amendment indicate that tomato plants may resist *T. absoluta* by involving ornithine metabolism, fatty acid biosynthesis, and PAMP-triggered immunity, respectively.

Phytohormones (ethylene, gibberellin, auxin, brassinosteroid, jasmonates, and salicylic acid) are involved in plant defense responses against insect attack and herbivory (Howe and Jander, 2008). Changes in their contents can activate signal transduction upon insect feeding or plant tissue wounding (Koch et al., 2016). The use of biochar has been shown to increase plant growth-mediated hormones (Farhangi-Abriz and Torabian, 2018). Our results that the expressions of BRI1, BAK1, DELLA, TCH4, MYC2, PYR/PYL, PP2C, and GID1 genes changed in tomato seedlings indicate that bamboo charcoal activates GA, ABA, brassinosteroid, and JA signaling. However, since we did not detect differential changes in phytohormone (or related metabolites), therefore, further research is needed to understand how bamboo charcoal may change the phytohormone profile of the tomato seedlings. Moreover, the increase in expression of genes enriched in MAPK signaling pathway such as ERECTA also suggests that bamboo charcoal can initiate signaling cascades in tomato leaves that help it maintain developmental integrity in biotic stress conditions. This proposition is based on the known role of ERECTA in maintaining plant phenotype and developmental integrity under different stress and microenvironmental scenarios (Douglas et al., 2002). In addition to phytohormone signaling, bamboo charcoal also regulated the expression of oxidative stress related genes such as GST, which plays roles against biotic and abiotic stresses in plants (Estévez and Hernández, 2020). Finally, our results indicate that bamboo charcoal activates the expression of different classes of TFs in tomato leaves to help the plant survive under attack. Earlier studies on different types of biochar have indicated that biochar-mediated plant growth under biotic stress is accompanied by the regulation of the expression of various TFs (Jaiswal et al., 2020). Different classes of TFs have been implicated in resistance against insect-pests e.g., ERF TFs enable tobacco plants to resist *Myzus periscae* and *Spodoptera litura* attack (Wu et al., 2020). Similarly, brassinosteroid related TF BIL1/BZR1 have been reported to help plant resist against insect feeding. Therefore, it can be understood that bamboo charcoal induced expression changes in a range of TF families, which in turn enabled tomato plants grow better and resist *T. absoluta* infestation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA898145.

Author contributions

LC and XL: conceptualization. QZ and AJ: methodology. TC and JW: software. JZ, JH, and ZZ: validation. MH: formal analysis. SZ: investigation. XR: resources. WD: data curation. YL: writing—original draft preparation. LC and XL: writing—review and editing. LC: visualization. YH: supervision. XL: project administration. XL and YL: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1101151/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Overall distribution of gene expression, **(B)** Principal component analysis, and **(C)** Pearson's correlation coefficient based on FPKM values of the differentially expressed genes be-tween CK and BC tomato leaves.

SUPPLEMENTARY FIGURE 2
qRT-PCR results of 15 selected genes in tomato. *Indicates significant differences be-tween the CK and BC ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

SUPPLEMENTARY TABLE 1
List of primers used for qRT-PCR analysis.

SUPPLEMENTARY TABLE 2
(A) Morphological performance of the tomato plants grown in three different coconut bran and bamboo charcoal combinations. **(B)** Probability of survival of *T. absoluta* (raw data).

SUPPLEMENTARY TABLE 3
List of differentially accumulated metabolites in tomato leaves grown in CK (100% coconut bran) and BC (30:1 v/v coconut bran and bamboo charcoal).

SUPPLEMENTARY TABLE 4
List of differentially expressed genes in tomato leaves grown in CK (100% coconut bran) and BC (30:1 v/v coconut bran and bamboo charcoal).

SUPPLEMENTARY TABLE 5
List of genes associated with different KEGG pathways that showed changes in the ex-pression between leaves grown in CK (100% coconut bran) and BC (30:1 v/v coconut bran and bamboo charcoal).

SUPPLEMENTARY TABLE 6
Expression changes in transcription factors between CK and BC tomato leaves grown in CK (100% coconut bran) and BC (30:1 v/v coconut bran and bamboo charcoal).

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EDITED BY
Xingyuan Men,
Shandong Academy of Agricultural Sciences, China

REVIEWED BY
Yu Shi,
Henan University, China
Xiaoyulong Chen,
Guizhou University, China
Jiatao Xie,
Huazhong Agricultural University, China

*CORRESPONDENCE
Zhuo Zhang
✉ lionkingno.1@163.com
Yong Liu
✉ haoasliu@163.com

†These authors have contributed equally to this work

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Rhodopseudomonas palustris PSB06 agent enhance pepper yield and regulating the rhizosphere microecological environment

Luyun Luo[1†], Pei Wang[2†], Diandong Wang[1], Xiaobin Shi[2], Jingwen Zhang[3], Zhixiang Zhao[4], Jun Zeng[5], Jingjing Liao[1], Zhuo Zhang[2*] and Yong Liu[2*]

[1]Yangtze Normal University, Chongqing, China, [2]Hunan Plant Protection Institute, Hunan Academy of Agricultural Sciences, Changsha, China, [3]College of Plant Protection, Hunan Agricultural University, Changsha, China, [4]Hainan Key Laboratory for Control of Plant Diseases and Insect Pests, Research Center for The Quality Safety and Standards of Agricultural Products in Hainan Academy of Agricultural Sciences, Plant Protection Institute of Hainan Academy of Agricultural Sciences, Haikou, China, [5]Institute of Applied Microbiology, Xinjiang Academy of Agricultural Sciences, Ürümq, China

The *Rhodopseudomonas palustris* (*R. palustris*) PSB06 can promote crop growth, as it maybe regulates microbial communities in plant root soil, soil physicochemical properties, thus creating a favorable habitat for the crop growth. However, there are few studies on the yields and rhizosphere microbial community of *R. palustris* PSB06 agent. In the study, the high-throughput sequencing was used to study the changes of rhizosphere soil bacterial community after PSB06 treatment. The results indicated *R. palustris* PSB06 agent increased the pepper yield by 33.45% when compared to control group, with better effect than other treatments. And it also significantly increased soil nitrogen concentration. *R. palustris* PSB06 agent had improved pepper rhizosphere bacterial α diversity and changed the community structure. Acidobacteria, Proteobacteria, Actinomycetes and Firmicutes were dominant phyla in all the pepper rhizosphere soil samples. The results showed that soil bacterial community were significantly positively correlated with pH ($R = 0.8537$, $P = 0.001$) and total nitrogen ($R = 0.4347$, $P = 0.003$). The nine significantly enriched OTU in *R.palustris* PSB06 treatment (PB) group belong to *Nitrososphaera* (OTU_109, OTU_14, OTU_18, OTU_8), *Lysobacter* (OTU_2115, OTU_13), *Arenimonas* (OTU_26), *Luteimonas* (OTU_49), and *Ramlibacter* (OTU_70) were significantly positively correlated with the total yield of pepper ($R > 0.5$, $P < 0.05$). Overall, our results provide a theoretical basis for studying the microbial regulation of *R.palustris* PSB06 on rhizosphere soil.

KEYWORDS

***Rhodopseudomonas palustris* PSB06, yield, diversity, bacterial community, rhizosphere**

Introduction

Microorganisms play an important role in the process of matter cycling and energy flow in the ecosystem and maintain the stability of soil ecosystem function (Guo et al., 2021). Soil microorganisms, phychemical properties and enzymes are important components of soil ecosystem and key factors in regulating soil microecological environment and function. Soil microorganisms can convert organic matter in soil into nutrients needed for plant growth (Yin et al., 2013). Root exudates play an important role in biogeochemical cycling, regulation of rhizosphere ecological processes, and plant growth and development. They can attract beneficial microorganisms and affect the assembly of rhizosphere microbiota, thus improving the ability of plants to adapt to the environment (Bulgarelli et al., 2012). Plant rhizosphere growth-promoting bacteria are a kind of beneficial microorganisms that can stably survive in plant rhizosphere and can promote growth and control soil-borne diseases. After entering the rhizosphere environment, they colonize the root surface by interacting with microorganisms in plants and soil to promote crop growth and control plant diseases.

Photosynthetic bacteria (PSB) are a group of prokaryotes that perform photosynthesis without oxygen production, widely distributed in soil, paddy fields, swamps, lakes, rivers, oceans and other places (Hohmann-Marriott and Blankenship, 2012). Photosynthetic bacteria have a mutualistic symbiosis with plants. They can significantly improve the available nutrients in soil by fixing nitrogen and solubilizing phosphate, and it also are excellent growth promoting bacteria in plant rhizosphere (Ndona et al., 2011; Wang et al., 2019a). *R. palustris* that belongs to PSB, which is considered to be the most metabolized bacteria. It can decompose various carbon and nitrogen sources, while it is also an important source of growth promoting bacteria in the rhizosphere of plants (Merugu et al., 2011; Wong et al., 2014). *R. palustris* is also a promising biofertilizer, which not only supplies plant nutrients through nitrogen fixation, but also increases the utilization rate of synthetic nitrogen fertilizer, thereby improving crop yield and soil fertility and promoting crop growth (Kornochalert et al., 2014; Nunkaew et al., 2014). *R. palustris* PS3 has a growth-promoting effect on a variety of crops (Wong et al., 2014; Lee et al., 2016). During leaf growth development, *R. palustris* PS3 inoculation can promote plant growth by enhancing nitrate absorption (Hsu et al., 2021). Arashida et al. (2019) co-cultured Bacillus subtilis and the purple non-sulfur bacterium *R. palustris* in nitrogen-free medium, and observed diazotrophic growth in the subculture. Foliar spraying of *R. palustris* can change the soil microbial community of stevia to promote plant growth (Xu et al., 2016). And *R. palustris* GJ-22 was proved that can also promote crop growth by producing IAA (Su et al., 2017). At the same time, studies on rice also showed that the inoculation of *R. palustris* could promote the plant growth, increase the crop yield and change the soil microbial community (Kantha et al., 2015; Luo et al., 2019). Therefore, *R. palustris* can effectively reduce the use of chemical fertilizers in agriculture, and has a broad application prospect in reducing the application of chemical fertilizers.

As an effective biological agent, *R. palustris* PSB06 has been registered as a pesticide. In this study, we compared the differences of rhizosphere bacterial communities of *R. palustris* PSB06 and other treatments in the field, analyzed the correlation between bacterial communities and the yield and physicochemical properties of pepper, and focused on the population differences of bacterial communities. Our studies will afford a theoretical basis for reveal the mechanism of *R. palustris* PSB06 affecting pepper yield and regulating rhizosphere environment.

Materials and methods

Experimental design

Field experiment was conducted in Maidi Village (112°28′54′′E, 29°29′52′′N), Yueshi Town, Huarong County, Yueyang City, Hunan Province from March 15 to June 18 in 2018. The pepper variety selected in this experiment was "Xiangyan 15", which was grown in large quantities in Hunan Province from Longping Seed Industry Company. The strain *Rhodopseudomonas palustris* (*R. palustris*) PSB06 (CCTCC No: M2012518) was used in the study with 10^7 cfu/g from Hunan Institute of Plant Protection. And the planting soil had a pH = 7.74, soil matter = 15.1 $\text{mg}\cdot\text{g}^{-1}$, available K = 122.70 $\text{mg}\cdot\text{g}^{-1}$, available P = 43.08 $\text{mg}\cdot\text{g}^{-1}$, total N = 1328.69 $\text{mg}\cdot\text{g}^{-1}$, total P = 864.50 $\text{mg}\cdot\text{g}^{-1}$, and total K = 1818.61 $\text{mg}\cdot\text{g}^{-1}$. The field experiment was carried out with five groups, one group is the soil samples before the experiment, and other four groups are the experimental treatment group. LB: soil samples before planting; PB: *R. palustris* PSB06 fermentation broth; MB: Farmhouse manure (2 $\text{ton}\cdot\text{mu}^{-1}$ of chicken manure); CB: Conventional fertilizer (51% Sanning compound fertilizer, N: P_2O_5:K_2O: 25:10:16:2 $\text{ton}\cdot\text{mu}^{-1}$, Hubei Sanning Chemical Co., Ltd.) and CKB: Fresh water. Each experimental treatment was repeated for seven times, with a total of twenty-eight plots, and fifty pepper plants were planted in each plot. The plots were arranged in completely random groups. The pepper was treated with *R. palustris* PSB06 agent at 7-leaf stage, with 30 mL per plant, and the root was irrigated once every 7 days for 3 times in total. After 30 days of field application, five-point sampling method was used to randomly select sampling points to collect the rhizosphere and surrounding soil of pepper. Soil samples of four pepper plants were collected in each site and mixed as one repeated soil sample.

Determination of pepper yield

Pepper fruits were collected since the first fruiting time after treatment, and collected every ten days and three times in total. The peppers were collected and weighed in each time, while the yield was recorded.

Determination of soil physiochemical properties

The pepper root surrounding soil was collected by shaking root method and then air-dried. The physiochemical properties of the air-dried soil samples were determined. The pH of the manure samples was measured in the aqueous extract (soil: deionized water = 1:2.5) using a multi-parameter water quality-monitoring instrument. Total potassium (TK, measured according to flame atomic absorption spectrophotometric method, GB 9836-1998), total nitrogen (TN, measured according to the modified Kjeldahl method, HJ/T 707-2014), total phosphorus (TP, measured according to the sodium hydrogen carbonate solution-Mo-Sb anti spectrophotometric method, HJ/T 704-2014), and organic matter (OM, measured according to the method for determination of soil organic matter, GB9834-1988) were measured by the Institute of Soil Science, Chinese Academy of Sciences (Nanjing, China).

DNA extraction, PCR amplification and high-throughput sequencing

The pepper rhizosphere soil samples were collected at the 7 day after the third irrigation. The roots were removed by shaking off the root method, and the roots were placed in a conical flask containing 100 mL 0.05 mol/L phosphoric acid buffer (pH = 7.0). Place four plants per conical flask and shake the conical flask from side to side to wash thoroughly the roots of the plants so that the soil is fully mixed into the buffer. Remove the cleaned root blocks from the conical flask with sterile forceps, then pour the remaining mixture into a 50 mL sterile plastic sterile centrifuge tube at a high speed of 13,000 rpm for 3 min to allow the soil to fully settle in the centrifuge tube and discard the supernatant. Then 50 mL centrifuge tube containing the soil sample was placed in a freeze-drying apparatus for freeze-drying. After freeze-drying, the soil samples were thoroughly ground with a sterile mortar, filtered through an 80-mesh sieve, and stored in a refrigerator at −20°C. 0.5 g of each sample was accurately weighed for DNA extraction that using the Fast DNA Spin Kit for Soil (MP Biomedicals, USA) according to the Kit instructions.

The total DNA concentration of samples were determined by NanoDrop 2000, with the A260/A280 value required between 1.8 and 2.0. The genomic DNA concentration of all samples was quantified to 30 ng·μL $^{-1}$ before amplification. Taking the total DNA of the sample as the template, Universal primers 515F (5 '-GTGCCAGCMGCCGCGGTAA-3') and 806R (5 '-GGACTACHVGGGTWTCTAAT-3') were used for PCR amplification of bacterial 16S rDNA fragments with 6 bp barcode (Wang et al., 2015). The PCR reaction system as follows: 5 μL 10×PCR buffer (containing 20 mmol·L^{-1} $MgCl_2$), 4 μL dNTP (10 mmol·L^{-1}), 1 U Taq DNA polymerase, 1 μL DNA template, and sterilized ddH_2O supplemented to 50 μL. PCR reaction conditions: pre-denaturation at 95°C for 10 min, after denaturation at 95°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 45 s, cycling for 35 times. Finally, it was extended at 72°C for 10 min and stored at 4°C at constant temperature. The purified PCR products were sent to Nanjing Puvekon Biotechnology Co., Ltd. (Nanjing, China) for sequencing. The clean reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA869895).

Data analysis

Raw sequence data reads were processed with an in-house pipeline (http://mem.rcees.ac.cn:8080). In brief, a separate sample was generated according to different barcodes and primers, allowing for one mismatch. Paired-end reads with at least 30 bp overlap were combined by the FLASH program (Magoč and Salzberg, 2011), and filtered by Btrim program with Quality Score <20 (Kong, 2011). Then we discarded the sequences with either an ambiguous base or <200 bp. The UPARSE algorithms were used to detecte and remove chimera sequences (Edgar, 2013). Low abundance OTUs (≤1 count) were eliminated from the OTU table. The bacterial representative sequences for each OTU were assigned to different taxonomic groups using the RDP Classifier database (Silva database 132 version). The resampled OTU table, which was obtained by resampled randomly with the lowest sequence number, was used for the subsequent analysis. The α-diversity was assessed using the Chao1, Observed_richness, Shannon and Inv_simpson index. Weighted principal coordination analysis (Weighted_PCoA) based on unifrac matrix, multi-response permutation procedures (MRPP), analysis of similarities (ANOSIM), and Adonis were used to test the bacterial community differences among the groups. The mantel test and canonical correspondence analysis (CCA) were used to analyze the relationship between environmental factors and bacterial communities. The key bacterial taxa responsible for discrimination between two groups were identified using linear discriminant analysis (LEfSe) with linear discriminant analysis (LDA) = 3.

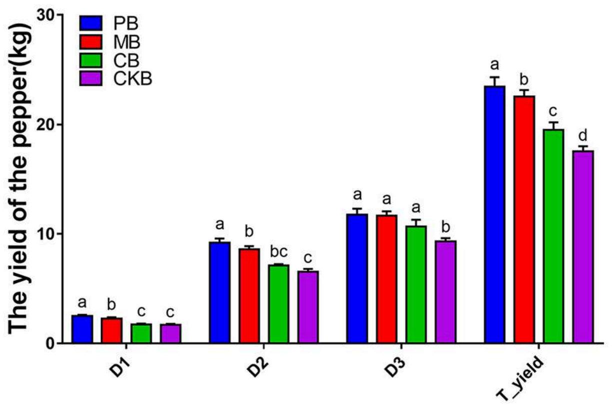

FIGURE 1
The total pepper yield and yield at different time points. a–d indicate the statistical diferences at $P < 0.05$ for one-way ANOVA.

Statistical analysis

The difference of α-diversity index and yield of pepper among different treatments were evaluated by one-way ANOVA after multiple comparisons based on Duncan algorithm using IBM SPSS for Windows (v.22.0) software. The results were presented as mean ± standard error (SE). The Student t test was used to assessed the difference between two groups of soil physichemical properties and relative abundance of top 30 genera, and statistical significant level was set at $P < 0.05$ by the software Microsoft Excel 2019. The spearman correlation coefficient was used to analyze the correlations between the core genera of the four treatment groups and yield, and the results were visualized using Cytoscape 3.6.0 software.

Results

Effects of *R. palustris* PSB06 agent on pepper yield in the field

The total yield and yield of each time points of pepper were calculated, and the results were shown in Figure 1. The yield of pepper in *R. palustris* PSB06 root-irrigation treatment (PB) and farm manure treatment (MB) groups was significantly higher than that in the control group when the pepper was collected at first and second sampling time ($P < 0.05$), and there was no significant difference between formal fertilizer treatment (CB) group and control group (CKB). But the yield of pepper in the experimental group was significantly higher than that in the control group at third sampling time ($P < 0.05$). The total yield of pepper in PB, MB and CB groups

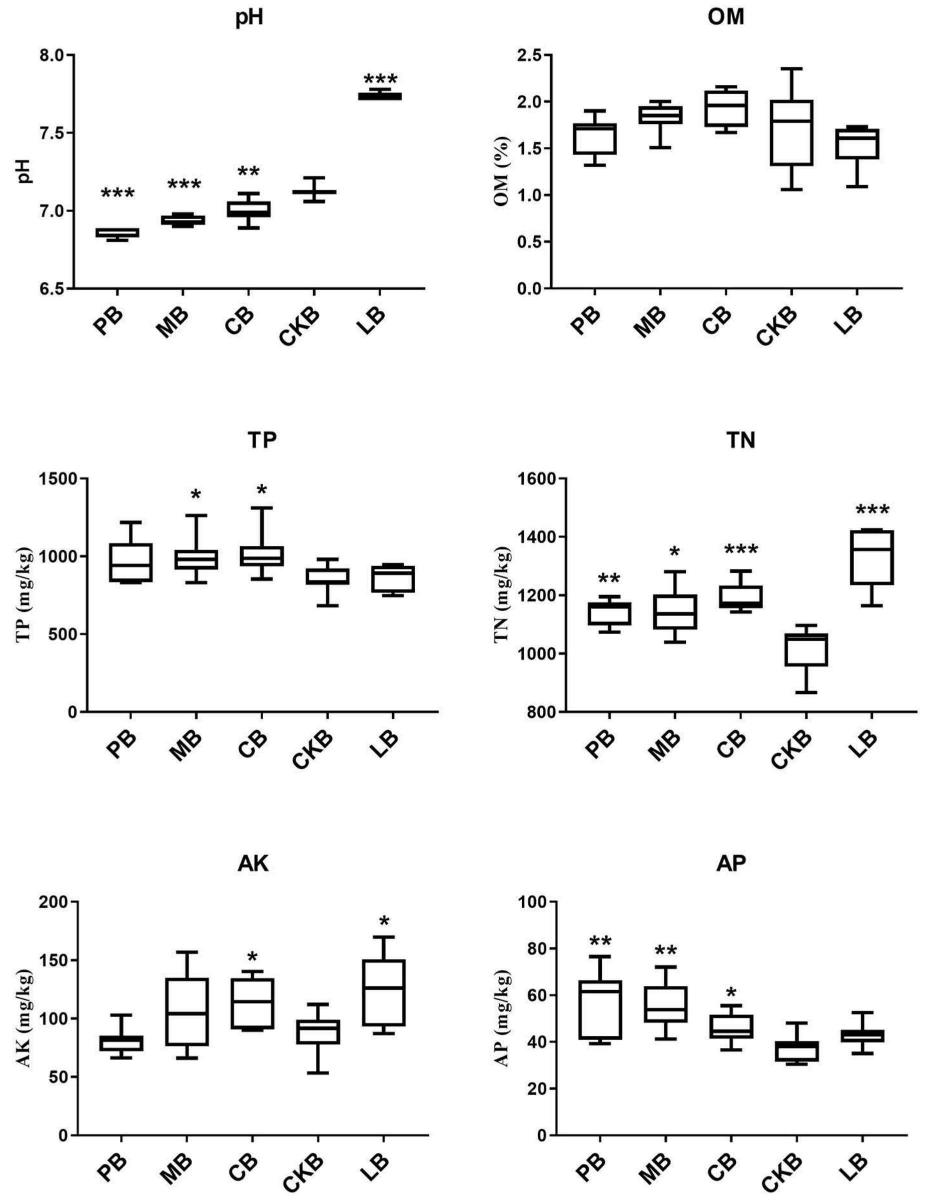

FIGURE 2
The physicochemical properties of different treatments. PB, *R. palustris* PSB06 root-irrigation treatment; MB, farm manure treatment; CB, formal fertilizer treatment; CKB, control treatment; LB, pre-planting soil sample group, $n = 7$. "*", "**", "***" indicate significant difference between treatments and control group, significance level at $P < 0.05$, $P < 0.01$, $P < 0.001$.

was significantly higher than that in control group ($P < 0.05$) and highest in PB group. Compared with the control group, the total yield of pepper in PB, MB and CB groups increased by 33.45, 28.44, and 11.03%, respectively.

Effects of PSB06 agent on soil physicochemical properties

Physiochemical properties of soil were measured before planting and 30 days after treatment, and the results were shown in Figure 2. Before planting pepper, the pH, total nitrogen (TN), available kalium (AK) values of pre-planting soil sample group (LB) were the highest and significantly higher than control group (CKB), but no significant difference at organic matter (OM), total phosphorus (TP), available phosphorus (AP) concentration. The pH of PB, MB and CB groups were significantly lower than those of CKB group ($P < 0.05$), and the pH of PB group was the lowest (pH = 6.9).

The contents of TN and AP in three experimental groups were significantly higher than those in CKB group (1011.86 mg/kg) ($P < 0.05$), the concentration of AP in PB group (56.78 mg/kg) was the highest, and the concentration of AP in PB group was 49.88% higher than control group. There was no significant difference in OM concentration between treatment and control group. The concentration of TP in CB and MB groups was significantly higher than that in control group ($P < 0.05$). The AP concentration of CB group was significantly higher than CKB, but there was no significant difference between PB, MB groups and CKB group.

TABLE 1 Summary of α diversity indices among different treatments.

Treatment	Chao1	Shannon	Inv_Simpson	Observed_richness
PB	2663.61 ± 124.8b	4.95 ± 0.04b	30.72 ± 1.99b	1360.57 ± 28.26b
MB	2385.3 ± 140.88b	4.26 ± 0.11c	17.62 ± 2.54b	1109.71 ± 50.03cd
CB	2592.23 ± 104.17b	4.7 ± 0.07b	27.87 ± 2.28b	1183.57 ± 31.29c
CKB	2421.51 ± 67.85b	4.19 ± 0.11c	17.73 ± 1.85b	979 ± 50.56d
LB	3772.52 ± 55.18a	6.49 ± 0.13a	247.44 ± 44.5a	2300.14 ± 72.02a

PB, *R. palustris* PSB06 root-irrigation treatment; MB, farm manure treatment; CB, formal fertilizer treatment; CKB, control treatment; LB, soil samples before transplant seedlings.
a, b, c, d indicate the statistical differences at $P < 0.05$ for one-way ANOVA. $n = 7$.

Effects of *R. palustris* PSB06 agent on rhizosphere bacterial community

A total of 1,648,432 high quality sequences were obtained from the raw data of 35 soil samples in the field experiment after a series of quality controls, with the sequence number ranging from 23,339 to 87,188. A total of 4861 OTU representative sequences were obtained from each group of samples by clustering at 97% similarity. The bacterial α diversity index of the pre-planting soil sample group (LB) and four treatment groups were shown in Table 1. The bacterial α-diversity index (Chao1, Observed_richness, Shannon, and Inv_Simpson index) of the PB group was significantly higher than other four treatment groups. The bacterial α-diversity index (Observed_richness, Shannon index) of the PB and CB group was significantly higher than control group (CKB) while no significant difference between MB and CKB group. The bacterial α-diversity index (Chao1 and Inv_Simpson index) of the PB and CB group was higher than CKB group but no significant difference among these groups.

A total of 4861 operational taxa (OTU) were identified from 35 soil samples in the field. The dominant phyla and class is shown in Supplementary Figure S1A. The dominant phylum in LB group were Proteobacteria (38.87%), Actinobacteria (11.68%), Thaumarchaeota (7.48%), and Bacteroidetes (7.82%), respectively. And Acidobacteria, Proteobacteria, Actinobacteria, and Firmicutes all were dominant phyla in the four groups of pepper rhizosphere soil samples. At the class level, the dominant populations were shown in Supplementary Figure S1B, and all OTUs are divided into 80 class. The dominant class in LB soil samples were α -proteobacteria, β -proteobacteria, γ -proteobacteria and δ -proteobacteria, Actinobacteria and Acidobacteria Gp6. The dominant class in the four groups pepper rhizosphere soil samples were α -proteobacteria, β -proteobacteria, γ -proteobacteria and Bacilli, respectively. At the phylum identification level, the dominant bacterial groups in the LB group (relative abundance greater than 4%) were Proteobacteria, Actinobacteria, Thaumarchaeota, Bacteroidetes and Acidobacteria. The dominant phyla in soil samples of PB group were Proteobacteria (58.47%), Actinobacteria (12.18%), and Firmicutes (9.46%), and Thaumarchaeota (Chigarchaea: 6.19%). The dominant phyla in the soil samples of the MB group were Proteobacteria (71.79%), Actinobacteria (11.56%), and Firmicutes (4.91%), while conventional fertilizer treatment (CB) group were Proteobacteria (54.57%), Actinobacteria (14.11%), Firmicutes (8.69%), and Bacteroidetes (14.11%). 7.22%), and Acidobacteria (5.48%). And in the control group (CKB), Proteobacteria (69.50%), Actinobacteria (12.60%), and Firmicutes (5.95%) were the dominant phyla.

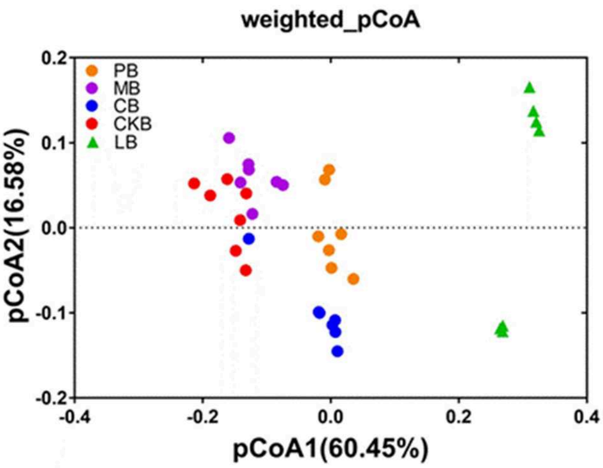

FIGURE 3
Principal coordinate analysis (PCoA) of bacterial communities among different treatments. PB, *R. palustris* PSB06 root-irrigation treatment; MB, farm manure treatment; CB, formal fertilizer treatment; CKB, control treatment; LB, soil samples before planting, $n = 7$.

At the genus level, the differences of the top 30 genera among the five groups were analyzed, and the results were shown in Supplementary Figure S2. The results showed that the relative abundance of *Flavobacterium, Gaiella, Gp6, Nitrososphaera, Phycicoccus* and *Arenimonas* in LB group was significantly higher than that in CKB group but significantly lower in *Aeromicrobium, Bacillus, Ensifer, Enterobacter, Factibacillus, Lechevalieria, Lysobacter, Nocardioides, Paenibacillus, Pseudomonas, Pseudoxanthomonas, Rhizobium, Shinella, Sphingobium, Streptophyta, Streptomyces, Variovorax*. The relative abundance of *Gaiella, Gp6, Nitrososphaera, Ramlibacter*, and *Arenimonas* in PB group was significantly higher than that in control group while lower in *Enterobacter, Nocardioides, Pseudomonas, Rhizobium, and Streptophyta*. The relative abundance of *Gaiella, Gp6* in MB group was significantly higher than that in control group while lower in *Marmoricola, Sphingomonas* and *Streptophyta*. The relative abundance of *Agromyces, Arthrobacter, Dyadobacter, Gaiella, Lysobacter, Nitrososphaera, Phycicoccus, Pseudoxanthomonas, Ramlibacter* and *Variovorax* in the CB group was significantly higher than that in control group while lower in *Ensifer, Enterobacter, Lechevalieria, Rhizobium* and *Sphingomonas.*

The differences of the rhizosphere soil bacterial community structure among soil samples were compared, and the results were shown in Figure 3 and Supplementary Table S1. Principal coordinate

TABLE 2 The mantel test result between environmental factors and bacterial communities based on Bray-Curtis and Jaccard distances.

Factors	R.BC	*P*.BC	R.JC	*P*.JC
pH	0.8537	0.001	0.727	0.001
AK	0.1049	0.134	0.0878	0.088
TN	0.4347	0.003	0.4122	0.001
TP	−0.0372	0.617	−0.0527	0.771
AP	−0.1018	0.884	−0.056	0.81
OM	0.07	0.26	0.1255	0.042

AK, available potassium; TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; OM, organic matter; BC, Bray-Curtis distance; JC, Jaccard distance; R.BC, correlation coefficient value of BC; *P*.BC, significance value of BC; R.JC, correlation coefficient value of JC; *P*.JC, significance value of JC.

analysis (PCoA) and dissimilarity analysis showed that there were significant differences in the bacterial community structure between the the pre-planting soil samples and the four pepper rhizosphere soil samples. The PCoA results indicated that the bacterial communities of the pre-planting soil samples and the four pepper rhizosphere soil samples were significantly separated while the bacterial communities of the four treatment groups were also significantly separated from each other. PCoA1 and pCoA2 accounted for 77.03% of the total variation. The results of dissimilarity analysis (MRPP, ANOSIM, and ADONIS) based on Bray-Curtis matrix showed that there were significant differences between LB and CKB group ($P < 0.01$), and the three experimental groups (PB, MB, CB) were also significantly different from CKB group ($P < 0.05$).

Correlation analysis of yield, physicochemical properties and bacterial community

The results showed that the total yield was correlated with pH (R = −0.651, $P < 0.001$) and TK (R = −0.4237, $P < 0.05$). In addition, TP was positively correlated with AP and TN, while pH was negatively correlated with TP, TN, and AP. Mantel test analysis was used to assess the relationship between bacterial community structure and environmental factors (Table 2). The results showed that soil bacterial community were significantly positively correlated with pH ($R = 0.8537$, $P = 0.001$) and TN ($R = 0.4347$, $P = 0.003$) (Supplementary Figure S3).

In addition, CCA was used to evaluate the soil bacterial community and physiochemical properties among all groups, as shown in Supplementary Figure S4. Soil OM, AP, pH, TN, and AP were significantly correlated with the bacterial community structure between the pre-planting soil samples group (LB) and four treatment group samples, and CCA1 and CCA2 accounted for 86.53% of the total variation. The Total (N, P), available (P, K), and pH were significantly correlated with the soil community structure, explaining 54.60% of the total variation, which CCA1 and CCA2 explained 31.96 and 22.64% of the variation, respectively.

The LefSe method was used to screen significantly enriched OTU in the three treatment groups and the control group, among which 12 OTU were enriched in the PB group, 19 OTU in the CB group and 4 OTU in the MB group (Figure 4). And 9, 5, 2 enriched OTU in the PB, CB, MB group were significantly positively correlated with the total pepper yields ($R > 0.5$, $P < 0.05$), respectively (Figure 4A). These OTU mainly belong to Thaumarchaeota, Proteobacteria, Actinobacteria, and Verrucomicrobia. The significantly enriched OTU in PB group belong to *Nitrososphaera* (OTU_109, OTU_14, OTU_18, OTU_8), *Lysobacter* (OTU_2115, OTU_13), *Arenimonas* (OTU_26), *Luteimonas* (OTU_49), and *Ramlibacter* (OTU_70). And the significantly enriched OTU in CB group belong to *Nitrososphaera* (OTU_18, OTU_109), *Lysobacter* (OTU_13), *Phycicoccus* (OTU_31) and *Terrimicrobium* (OTU_68) while *Acinetobacter* (OTU_55, OTU_74) in the MB group.

Discussion

It is an environmentally friendly approach to improve the crop yield and soil environment by introducing beneficial microbes to the agriculture ecological system. Previous study also indicated that soil microbial inoculants can enhanced nutrient uptake and stimulated plant growth accumulation after whole-inoculation procedures (Wang et al., 2019a). In our study, the total yield of pepper treated with *R. palustris* PSB06, farm manure and conventional fertilizer was significantly higher than control group ($P < 0.05$), which increased by 33.45, 28.44, and 11.03%, respectively. And it indicated that *R. palustris* PSB06 inoculations significantly increased pepper yield, with better yield increase than other treatments. In previous studies, *R. palustris* is also a promising biofertilizer, which improving crop yield and soil fertility by supplies plant nutrients through nitrogen fixation and increases the utilization rate of synthetic nitrogen fertilizer (Kornochalert et al., 2014; Nunkaew et al., 2014). *R. palustris* PS3 has a growth-promoting effect on a variety of crops (Wong et al., 2014; Lee et al., 2016). In agricultural production, the application of *R. palustris* PSB06 as a biofertilizer to reduce or replace the use of manure and chemical fertilizers will effectively reduce the emission of pollutants in the environment and the environmental safety problems caused by the overuse of chemical fertilizers to a certain extent.

Soil microbes play an important role in agroecosystems by mediating biogeochemical and nutrient transformation (Banerjee et al., 2018; Zhu et al., 2018). The rhizosphere is the most active area in the plant-microbe interactions system. Some studies have evaluated the influences of different microbial inoculants on soil properties and plant nutrient uptake under controlled conditions (Qiao et al., 2019; Wang et al., 2019b, 2021a). The composition and assembly process of rhizosphere bacterial communities may be affected by different rhizosphere environments. In this study, the soil pH of *R. palustris* PSB06, farm manure and conventional fertilizer group was significantly lower than control group, and the total nitrogen concentration was increased by 13.22, 12.92, and 18.19%, respectively. Previous studies indicated that each environmental variables may have different driving effects on community assembly and composition (Lee et al., 2017; Wang et al., 2017; Zhao et al., 2017). Conventional fertilizers and farm fertilizers contain a large amount of nitrogen and phosphorus elements, which were directly absorbed and utilized by plants when applied to the environment. *R. palustris* PSB06, as a bacteria with nitrogen fixation function, it can indirectly promote the pepper growth and development by producing nutrients needed by plants through biological nitrogen fixation. In addition, we also analyzed the association between rhizosphere bacterial microbiota and the corresponding environmental factors.

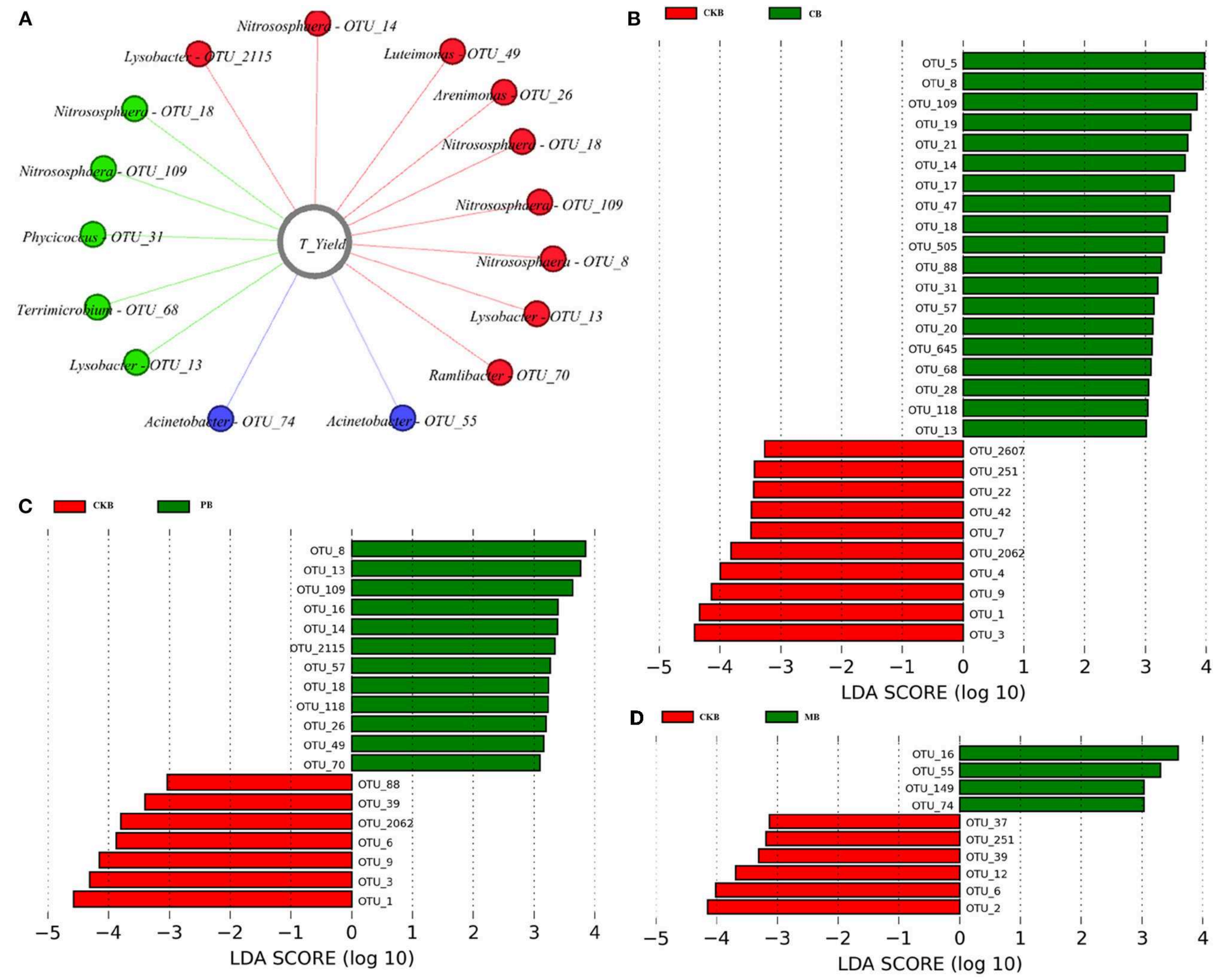

FIGURE 4
LefSe analysis between three treatment groups and the control group. **(A)** The enriched significantly OTU in the PB, CB, MB group were significantly positively correlated with the total yield of pepper ($R > 0.5$, $P < 0.05$), respectively. **(B)** The significantly enriched OTU between CB and CK group. **(C)** The significantly enriched OTU between PB and CK group. **(D)** The significantly enriched OTU between MB and CK group.

The results showed that soil bacterial community were significantly positively correlated with pH ($R = 0.8537$, $P = 0.001$) and TN ($R = 0.4347$, $P = 0.003$). It indicated that the soil TN and pH significantly affected rhizosphere bacterial community composition, and further demonstrated the function of nitrogen in microbial community changes and pepper growth and development. The changes in soil chemical factors due to *R. palustris* PSB06 inoculation, such as nitrogen and pH, were the dominant factors explaining the succession of the resident community. Kuramae et al. (2010) also reported that soil pH significantly altered the microbial secondary succession, the soil pH in inoculated treatments significantly differed from that in non-inoculated soil. Wang et al. (2019a) found that the phosphate-solubilizing bacteria possess the ability to produce organic acid and release nutrient contents, thus leading to a decrease of the soil pH and changes in the related nutrient contents. In the present study, the concentration of TN were significantly increased but slight difference among available nutrients compared to the control group after the PSB06 inoculation application. Previous study also showed that the addition of bacterial growth medium had a very limited effect on soil available nutrients (Wang et al., 2021b). As a efficient and ecofriendly soil management strategy, microbial inoculants are applied for improving crop productivity and soil properties by colonizing the rhizosphere and increasing nutrient availability to the host plant (Yilmaz and Sönmez, 2017; Berg et al., 2020; Pagnani et al., 2020).

Soil microorganisms are the main drivers of soil ecosystem functioning (Zhong et al., 2020). However, the native soil microbial community is sensitive to exogenous disturbances and natural climate change (Hartmann et al., 2015; Suleiman et al., 2016). Human disturbance of farmland soil can greatly affect crop rhizosphere processes, especially rhizosphere microbial communities (Mariotte et al., 2018). The importance of the root-associated microbial community for plant growth and development has been widely recognized (Wagner et al., 2014; Debenport et al., 2015). Previous study suggested that invasion by a single strain may change microbial community composition and function, and diversity determines the outcome of biotic invasions (Mallon et al., 2018). Mawarda et al. (2020) also indicated that the deliberate release of microbial inoculants may affect resident microbiome interactions. Dissimilarities among rhizosphere bacterial community compositions at different treatments indicate that changes in the rhizosphere environment occur after factitious interference. In our study, principal coordinates analysis (PCoA) demonstrated that bacterial community structure between the experimental group and control group after application, indicating that *R. palustris* PSB06 irrigation changed the bacterial community structure in rhizosphere soil. In addtion, the α-diversity of rhizosphere bacterial community at *R. palustris* PSB06 group was significantly higher than control group, reflecting the application on the evolution of rhizosphere bacterial community.

Soil-resident microbial communities are frequently subjected to biotic disturbances, including beneficial microbial inoculants and harmful pathogens, which can change microbial community succession, composition, and diversity (Xiong et al., 2017; Lourenco et al., 2018). In our study, we defined the taxonomic structure of the pepper root microbiota, which mainly comprising Acidobacteria, Proteobacteria, Actinomycetes, and Firmicutes. Acidobacteria and Acidobacteria are the dominant phyla in rhizosphere soil and widely distributed in soil around the world, indicating that pepper rhizosphere bacteria also follow the general rule of bacterial community establishment (Delgado-Baquerizo et al., 2018). Acidobacteria can offers efficient carbon and nitrogen cycling from soil organic matter, while Proteobacteria can release nutrients from complexes of organo-mineral that facilitate plant growth (Lugtenberg and Kamilova, 2009; Eilers et al., 2010). Actinobacteria are generally defined as copiotrophic bacteria, while Acidobacteria are oligotrophic bacteria (Dai et al., 2018). Compared with the control group, PSB06 treatment group decreased the relative abundance of Proteobacteria and increased the relative abundance of Firmicutes. Wang et al. (2021b) found that the relative abundances of families like Xanthomonadaceae significantly increased after PSB treatments application, suggesting that the introduction of PSB changed specific resident microbial populations. The microbial invasions frequently usually start with a dominating microbial population and have an impact on the native soil microbiome (Mallon et al., 2018). Previous studies have attempted to evaluate the impacts on the microbial community in the rhizosphere of the introduction of plant-growth-promoting rhizobacteria (PGPR), it indicated that microbial inoculants may alter the resident community composition by causing resource competition, synergistic effects, and antagonistic effects (Zhang et al., 2019; Zhuang et al., 2021). These changes in the abundances of some taxa after the initial disturbance due to microbial inoculation may be a result of competition for resources in the soil (Krause et al., 2014).

Understanding the interactions among microbial taxa and environmental factors can reveal the complex microbial community structure and detect potential keystone species (Wang et al., 2015; He et al., 2017). Therefore, we screened the enriched OTUs in different treatments and analyzed their correlation with yield. The results showed that the PB group have more positive OTUs than other treatments and control group. The nine significantly enriched OTU in PB group belong to *Nitrososphaera* (OTU_109, OTU_14, OTU_18, OTU_8), *Lysobacter* (OTU_2115, OTU_13), Arenimonas (OTU_26), *Luteimonas* (OTU_49), and *Ramlibacter* (OTU_70) were significantly positively correlated with the total pepper yield ($R > 0.5$, $P < 0.05$). The yield improvement may be the result of microbial-crop interaction, our results indicated that cooperative microbial interactions may play an critical role in soil microbial assembly and may benefit plant growth and development. The functional study of these bacteria is also the focus of our next research. So, isolation and functional verification of rhizosphere microbiota is necessary for future work, especially for potential keystone species. It will help further our understanding of this microecosystem in the crop rhizosphere.

Conclusions

R. palustris PSB06 treatment increased the pepper yield and improved the rhizosphere soil microbial environment by increasing the bacterial alpha diversity and changing the rhizosphere bacterial structure, which created a more healthy soil environment for pepper growth.

Data availability statement

The data presented in the study are deposited in the SRA repository, accession number PRJNA869895. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA869895.

Author contributions

LL, PW, and ZZ performed the experiments and analyzed the data. ZXZ, JWZ, and XS prepared the figures and tables. LL, YL, and DW conceived and designed the experiments. JZ and JL analyzed the data, authored or reviewed drafts of the manuscript, and approved the final draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1125538/full#supplementary-material

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EDITED BY
Xingyuan Men,
Shandong Academy of Agricultural Sciences, China

REVIEWED BY
Wenhao Hu,
Zhejiang A&F University, China
Yuan-Xi Li,
Nanjing Agricultural University, China

*CORRESPONDENCE
Chao-Dong Zhu
✉ zhucd@ioz.ac.cn

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Optimizing low-cost sampling of pollinator insects in oilseed rape fields

Xiao-Yu Shi[1], Michael Orr[1], Arong Luo[1], Ming-Qiang Wang[2], Pengfei Guo[3], Qing-Song Zhou[1], Zeqing Niu[1], Huijie Qiao[4], Yi Zou[5] and Chao-Dong Zhu[1,6,7]*

[1]Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [2]CAS Key Laboratory of Mountain Ecological Restoration and Bioresource Utilization and Ecological Restoration and Biodiversity Conservation Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, Beijing, China, [3]College of Pharmacy, Guizhou University of Traditional Chinese Medicine, Guiyang, China, [4]Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [5]Department of Health and Environmental Sciences, Xi'an Jiaotong-Liverpool University, Suzhou, China, [6]State Key Laboratory of Integrated Pest Management, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [7]College of Life Sciences, University of Chinese Academy of Sciences, Beijing, China

Insects are key pollinators to ecosystem function, but much work remains to determine the most cost-effective, reliable scheme to monitor them. Pan traps (PT) and flight interception traps (FIT) are two of the most popular insect sampling methods used. However, their relative sampling performance and cost is poorly known for agroecosystems in China. We conducted a study across 18 oilseed rape fields in smallholder farmland in Zhejiang, China using these two traps. Our results showed that a single FIT had a greater sampling efficiency (more individuals and higher species richness) than a single PT, but controlling for cost, four PTs (the cost for four PTs is close to one FIT) showed a greater sampling efficiency than FITs. PTs collected more small-bodied individuals while FITs and PTs did not significantly differ in terms of monitoring pollinator insects with large body size. When exploring whether semi-natural habitat embedded in the agricultural landscape affected these results, results from both trap types shows that semi-natural habitat had a significant positive impact on wild pollinator diversity and rarefied species richness. Future studies that examine the effects of agricultural landscape on the wild pollinator community should combine PTs with netting or other active methods for long-term wild pollinator monitoring strategies.

KEYWORDS

pan trap, window trap, flight interception trap, mass-flowering crop, smallholder farmland, pollinator monitoring, pollinator diversity

Introduction

Insect pollinators provide important ecological services for crops globally (Klein et al., 2007; Aizen et al., 2009), but recent reports of decline make their future uncertain (Potts et al., 2010, 2016; Rhodes, 2018; LeBuhn and Vargas Luna, 2021). The decline of pollinators in the agroecosystems may lead to yield losses in pollinator-dependent crops (Steffan-Dewenter et al., 2005). To better conserve insect pollinators and maintain their pollination services, it is necessary to monitor their population dynamics and community composition using solid sampling methods (Howlett et al., 2009; Westerberg et al., 2021), as otherwise recommendations and subsequent conservation actions could prove ineffective.

Insect monitoring is generally done through a combination of active and passive collection methods. Active methods are relatively straightforward, involving either direct specimen collection or observation, and are best paired with passive sampling to ensure a full accounting of local biodiversity (Gibbs et al., 2017; Templ et al., 2019; Portman et al., 2020; Prendergast et al., 2020). Passive methods are more complicated, carried out through various types of traps (Prendergast et al., 2020). Notably, it is exceedingly important to test passive traps across settings, to understand their biases and also ensure that they are used in a responsible manner that does not potentially cause decline of local susceptible species (Gibbs et al., 2017; Portman et al., 2020).

Various passive sampling traps have been developed for pollinating insects, including pan trap (Cane et al., 2000; Westphal et al., 2008) and flight interception trap (Howlett et al., 2009). Pan traps (PTs) are colored containers filled with liquid that attract flower-visiting insects (Cane et al., 2000; Westphal et al., 2008); they have been widely applied in previous studies for pollinator biodiversity monitoring due to their cost-effectiveness (Zou et al., 2017; Wu et al., 2018; Larkin and Stanley, 2021; Shi et al., 2022a). However, as an attractant-based sampling trap, they have inherent biases (Cane et al., 2000). PT's sampling performance may be affected by surrounding flowering plants (Steven et al., 2003; Baum and Wallen, 2011; Westerberg et al., 2021), especially when deployed in mass-flowering crops, and tend to be biased toward pollinators with similar traits, for instance, ground nesters (Roulston et al., 2007), or those from the family Halictidae. Flight interception traps (FIT) are non-attractant traps and might result in different catches from PTs (Lamarre et al., 2012; Mesa et al., 2013). FITs are essentially transparent panes used to direct flying insects into collection traps held below (Knuff et al., 2019; González et al., 2020).

Some studies have previously compared PT and FIT efficacy, but these are largely outside of pollinator-dependent agroecosystems and have largely been conducted in just temperate areas (Kehinde and Samways, 2012; Cunningham et al., 2013; Rader et al., 2014). Additionally, sampling methods may be biased toward pollinators with different functional traits (Prendergast et al., 2020). Body size is important functional traits for pollinators and larger pollinators have been found to provide superior pollination services (Huda et al., 2015; Jauker et al., 2016). While some studies have explored the impacts of body size for trapping (McCravy et al., 2019; Krahner et al., 2021; Thompson et al., 2021), PTs and FITs have yet to be explicitly compared as such.

Habitat type can also strongly influence trapping results. In agricultural landscapes, semi-natural habitats (forest, shrub and grassland) can support the local wild pollinator communities through offering floral resources and nesting locations (Garibaldi et al., 2020; Raderschall et al., 2021; Shi et al., 2021). Consequently, it is necessary to account for habitat types in agroecosystems from a broad array of climates and crops, as otherwise the generality of best practices for trapping may be limited to overstudied temperate environments.

In this study, we collected wild pollinators using PTs and FITs in 18 oilseed rape (*Brassica napus* L.) fields in the smallholder agricultural landscapes in subtropical China. Oilseed rape requires insect pollination for optimal yield (pollinator-dependent) (Zou et al., 2017). Thus, monitoring pollinator diversity for oilseed rape is essential for agricultural production (Ouvrard et al., 2019). However, our knowledge of best practices for monitoring in oilseed rape is limited. In conducting long-term monitoring, financial (money to make traps) and labor costs (effort in assembly and deployment) must be considered. Our aims were to (1) assess the pollinator diversity for oilseed rape in these areas; (2) compare the sampling performance of these two trap types under the same budget, accounting for wild pollinator abundance and species richness. We further explore whether these two sampling methods are biased toward pollinators' body size; (3) To account for the effect of agricultural landscape, we also collected in a range of situations involving varying levels of semi-natural habitat. Thereby, we provide a better framework for understanding and monitoring crop pollinator diversity in the under-studied subtropics of East Asia.

Methods

Study sites and land use analysis

This study was conducted in oilseed rape fields in Kaihua County, Zhejiang Province, China. Kaihua County was chosen for its small field sizes due to montane terrain (Lou et al., 2019). Thereby, these landscapes well represent smallholder plantations. In the early spring, oilseed rape is the most dominant crop there. Later in May, oilseed rape will be harvested and rice will be cultivated in the same field (oilseed rape-rice rotation). Oilseed rape production is essential for local smallholder farmers' livelihood since many farmers use oilseed rape as their cooking oil sources rather than for sale. Oilseed rape pollinators sampling started at the end of February and ended in late April in 2022, covering the whole flowering season of oilseed rape. In total, 18 research sites were selected with one field each (Figure 1). All of the fields were managed using smallholder farming practices (< 2 hectares) (Lowder et al., 2016). The minimum distance between two sampling sites was 1.9 km with montane terrain between them, exceeding the average foraging distance of many insect pollinators (Chifflet et al., 2011). Semi-natural habitat is defined as the habitat in the agricultural landscape where non-crop plants grow (Holland et al., 2016). Semi-natural habitats in this study include forest, shrub and grassland that embedded in the agricultural landscape and water bodies, like rivers and streams were not included in the analysis, as in prior studies in China (Zou et al., 2017; Shi et al., 2021, 2022b) and abroad (Papanikolaou et al., 2017; Zou et al., 2017; Shi et al., 2021). The land-use data (forest, shrub, grassland and other) in the 1000 m radius for the focal oilseed rape field was collected using ground verification methods (Liu et al., 2016; Zou et al., 2017) in the spring of 2022. We selected a 1000 m radius for our landscape analysis as this is the foraging range for many pollinators (Zurbuchen et al., 2010; Chifflet et al., 2011) and the strongest impact of agricultural landscape on pollinator diversity has been shown at a scale of 1000 m radius (e.g., Zou et al., 2017). The proportion of semi-natural habitat in each site was calculated in Arcmap 10.8.

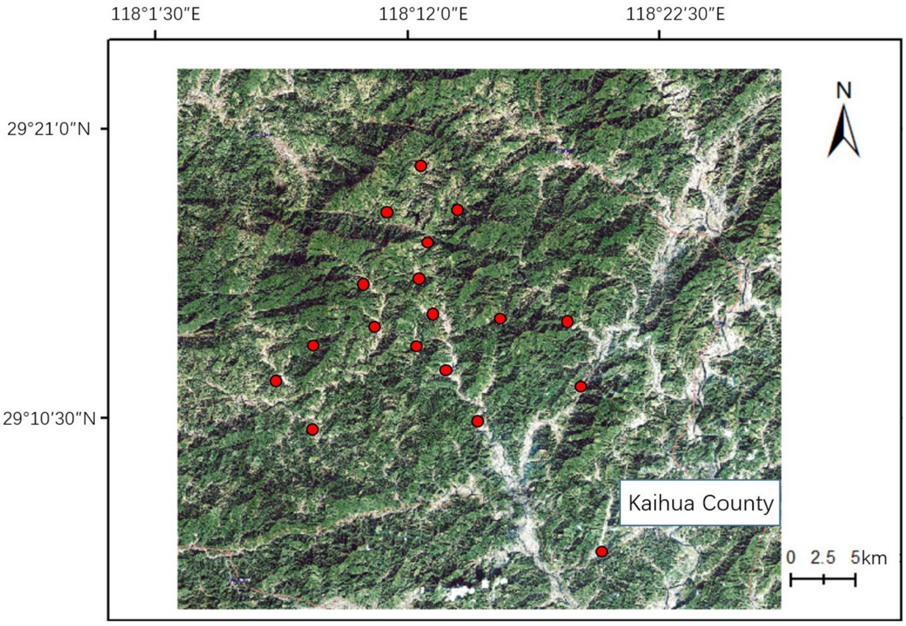

FIGURE 1
The 18 research sites located in Kaihua County, Zhejiang Province (China).

Pollinator sampling methods

FITs and PTs were deployed in one focal oilseed rape field at each sampling site. Each FIT was composed of a transparent acrylic plate (55*50 cm and 3 mm in thickness) fixed on two wooden sticks using plastic cable ties. Under the acrylic plate, a white plastic tray (60*43 cm and 11 cm in depth) was fixed to the sticks using metal wires. A PT array was made of three plastic cups (450 ml with diameter of 8.3 cm) with UV white, blue and yellow color, fixed on a stick about 1.5 m in height using metal ring cupholders. For both interception trap and PTs, small holes were drilled near the top of the containers for rainwater drainage. Photos of the two trap types can be found in Figure 2. The cost of manufacturing 18 FITs was 1450 RMB, including acrylic plates (650 RMB), plastic tray (630 RMB), wooden sticks (90 RMB), and iron wire and plastic cable ties (80 RMB). The money cost of manufacturing 72 PTs was 1410 RMB, including plastic cups (500 RMB), paint (550 RMB), and wooden sticks (360 RMB). The money costs of manufacturing one FIT (80.6 RMB) and four PTs (78.3 RMB) were close. In addition, the human labor for manufacturing two trap types were close (e.g., labor hours for both traps were around 16 h in 2 days). Thus, in each focal oilseed rape field, four PT sets were placed ~1 m from the field edge and one FIT was deployed, along a randomly-selected field edge for intercepting pollinators visiting the focal oilseed rape field. As two passive traps, FIT and PT sampling does not involve in intensive labor, which is different sweeping net that involving intensive labor work from experienced personnel. Overall, monetary and labor costs for conducting this pollinator sampling project were both low and feasible.

Samples were collected and traps reset every seven days, resulting in 52 sampling days per site. The collected samples were stored in the refrigerator (-20°C) for further species identification. European honeybees (*Apis mellifera*), as managed non-native pollinators in China, were excluded from this study. All insect samples were sorted to morpho-species and then identified to species or morphospecies by taxonomists. Pollinators were classified as large (body length > 12 mm) and small (body length < 12 mm) insects according to Albrecht et al. (2007). Hence, butterflies, carpenter bees, bumblebees, wasps, one longhorn bee (*Eucera floralia*), two digger bees (*Anthophora villosula* and *Anthophora plagiata*), two scoliid wasp species (Scoliidae sp1 and Scoliidae sp2) and one large hoverfly (*Phytomia zonata*) were grouped as large-bodied pollinators and the rest pollinators were grouped as small-bodied pollinators (Supplementary Table S1).

Statistics analysis

Samples from each site caught using the same sampling method were pooled to have a robust dataset. Linear mixed models were used to compare the differences in wild pollinator abundance, diversity and species richness between two trap types, of which study site was the random factor. The abundance of the three most abundant species (*Eucera floralia*, *Apis cerana* and *Gametis jucunda*), as well as the abundance of large and small pollinators between the two trap types were also compared *via* linear mixed models. Species richness was rarefied to 31 individuals which is the lowest sample size using FITs and PTs among all sites. Abundance was calculated using the total amount of pollinator individuals divided by sampling days (e.g. number of individuals per sampling days). We firstly compared catches of one set of FITs with a set of PTs, then with single PTs.

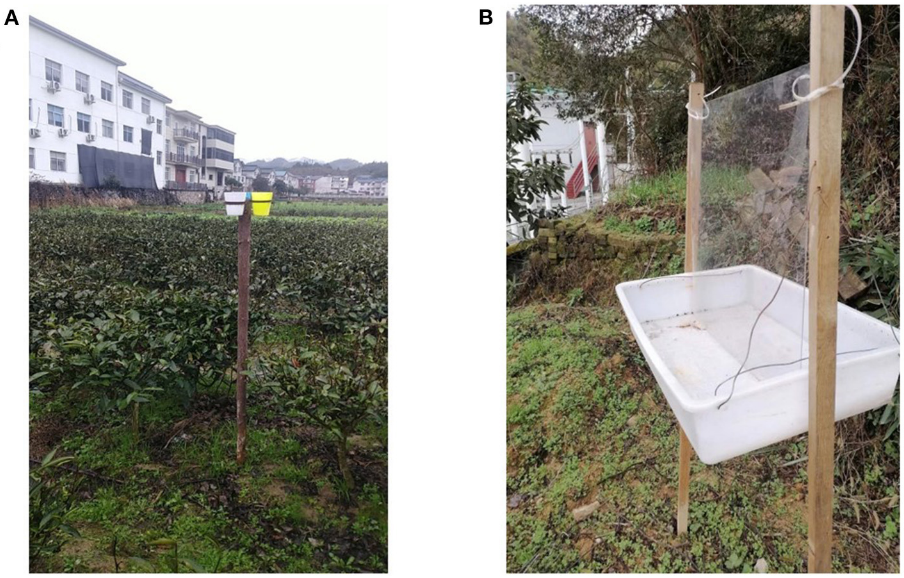

FIGURE 2
Examples of pan trap **(A)** and flight interception trap **(B)** setups.

In order to check whether or not samples from FITs and PTs had similar species compositions, we calculated the beta-diversity based on each site and different traps (i.e,. one FIT vs. 4 PTs from each site). To compare the pollinator community composition, we used Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance. The Bray-Curtis distance matrix was calculated as an index for beta-diversity, and is relatively robust to sampling size (Ricotta and Podani, 2017). We then used Principal Coordinate Analysis (PCoA) to visualize our results. ANOSIM was conducted with 9999 permutations to analyze the dissimilarity between pollinator communities in PTs and FITs. We also conduct indicator species analysis to check the species that were more often collected by one trap type.

Multiple linear regression was used to investigate the effect of the proportion of semi-natural habitats in on wild pollinator abundance and rarefied species richness. The proportion of semi-natural habitats at 1000 m was used as our response variable. To check whether semi-natural habitats had consistent effects on samples from different traps, interactions between proportion of semi-natural habitats and trap types were added as an explanatory variable. In the case of no interaction effect (and this is our case, see "Results"), analysis between semi-natural habitats and pollinator variables were conducted separately for FITs and PTs. We checked the heteroscedasticity for all linear regression models. To check for spatial autocorrelation, we calculated Moran's I of model residuals (Gittleman and Kot, 1990) and we did not detect any significant spatial correlations in any of the analyses in this study ($p > 0.05$).

All statistical analyses were conducted in R 3.5.2 (R Core Team, 2016). To calculate rarefied species richness at each site ($n = 31$, which was the least number of sampled wild pollinator using FITs and PTs among all sites), we used the package "vegan" (Oksanen et al., 2019). To produce rarefaction extrapolation curves, we used the function iNEXT in the package "iNEXT" (Hsieh et al., 2016). Function "lme()" packages "nlme" (Pinheiro et al., 2017) was used to conduct the linear mixed model. Function "anosim ()" in the "vegan" package (Oksanen et al., 2019) was used to compare the differences in wild pollinator community composition in FITs and PTs. Function "multipatt" in the package "indicspecies" was used to conduct indicator species analysis (De Caceres et al., 2016). Breusch-Pagan test using "bptest ()" function in R package "lmtest" (Hothorn et al., 2015) was used to check the heteroscedasticity. Package "ape" (Paradis and Schliep, 2019) was used to check the Moran's I value.

Results

In total, 2,970 (53 species) wild pollinator specimens were collected by FITs (1,230 individuals; 33 species) and PTs (1,740 individuals; 50 species) (species list see Supplementary Table S1 and rarefaction curve for PT and FIT see Supplementary Figure S1). The five most abundant wild pollinator species sampled by FITs were *Eucera floralia* (226), *Apis cerana* (203), *Gametis jucunda* (158), *Pieris rapae* (152) and *Halictus aerarius* (62). The five most abundant wild pollinators collected by PTs were *E. floralia* (254), *G. jucunda* (161), *Xylocopa tranquabaroroum* (146), *A. cerana* (140), and *Lasioglossum* sp (133) (Supplementary Table S1). Single FITs catch significantly more individuals per sampling day (1.31 ± 0.14) and rarefied species (9.22 ± 0.46) than single PTs (0.47 ± 0.04 per sampling day and 2.61 ± 0.12 species) ($p < 0.05$) while four PTs collected significantly higher individuals per sampling day (1.86 ± 0.14) and species (10.43 ± 0.46) than single FITs ($p < 0.05$) (Figures 3A, B). There was no significant difference in the abundance per sampling day of the three dominant pollinator species *A. cerana*, *G. jucunda* and *E. floralia* between PTs and FITs ($p > 0.05$). *Lasioglossum* sp.1, *Ceratina japonica*, *Lasioglossum* sp2, *Chrysomya megacephala*, *Lasioglossum* sp were five species caught

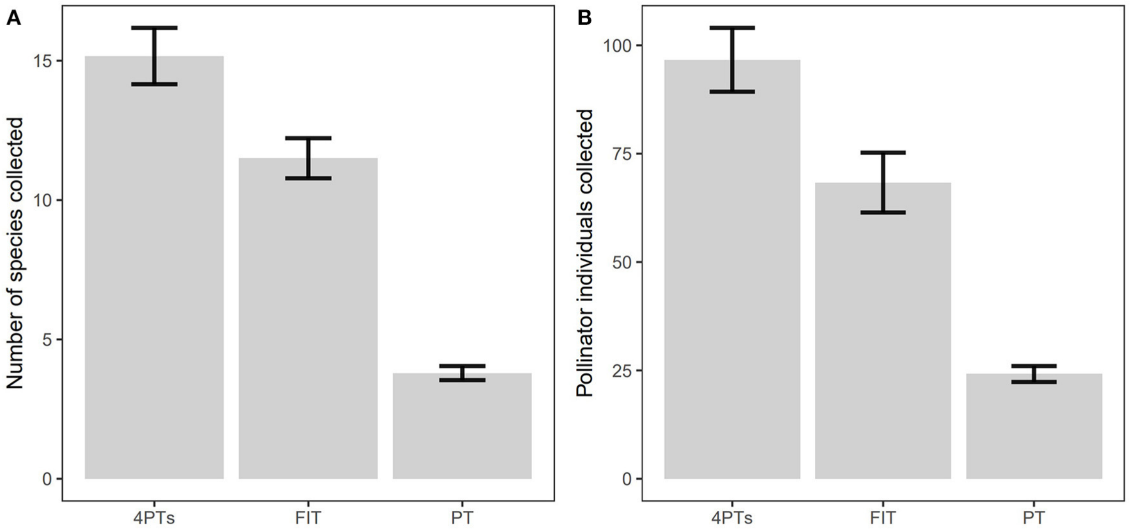

FIGURE 3
Number of species **(A)** and **(B)** pollinator individuals collected by a FIT, a single PT and four PTs combined (4PTs).

significantly more often in the PTs ($p < 0.0.05$) while no species caught significantly more often in the FITs.

The proportion of each wild pollinator species (log-transformed) in the insect in PTs and FITs was significantly correlated ($r^2 = 0.70$, $p < 0.001$) (Figure 4). Wild pollinator abundance measures collected using FITs and PTs were not significantly correlated ($p > 0.05$), but species richness and diversity in FITs and PTs were significantly positively correlated ($p < 0.05$). The analysis of similarities (ANOSIM) test recovered significant differences between the pollinator communities collected by FITs and PTs overall ($R = 0.2081$; $p < 0.001$; Figure 5). There was no significant difference in the abundance and relative abundance of large-sized pollinators in FITs and PTs ($p > 0.05$) (Supplementary Figure S2). The abundance of small pollinator insects in PTs was significantly higher than in FITs ($p < 0.05$) while there was no significant difference in relative abundance ($p > 0.05$) (Supplementary Figure S2).

Semi-natural habitat in both small-scale (500 m) and large-scale (1000 m) had positive effects on wild pollinator rarefied species richness and diversity ($p < 0.05$), while semi-natural habitat had no significant impact on wild pollinator abundance ($p > 0.05$) (Table 1). There was no significant effect of sampling type on wild pollinator abundance, rarefied species richness or diversity ($p > 0.05$) (Figure 6; Table 1). There was also no significant effect of interactions between sampling types and semi-natural habitats on wild pollinator abundance, rarefied species richness and diversity ($p > 0.05$) (Figure 6; Table 1). At the 1000 m radius scale, semi-natural habitat had no significant effect on wild pollinator abundance collected by FITs (slope = 0.09, intercept = 1.25, $p > 0.05$) or PTs (slope = 0.29, intercept = 1.65, $p > 0.05$) (Figure 6A). Semi-natural habitat had a significant positive impact on wild pollinator rarefied species richness collected by both FITs (slope = 10.43, intercept = 1.53, $p < 0.001$) and PTs (slope = 8.35, intercept = 4.28, $p < 0.05$) (Figure 6B). Semi-natural habitat in the agricultural landscape had significant positive impact on wild pollinator diversity collected by FITs (slope = 1.41, intercept = 0.95, $p < 0.001$) and had marginally significant effect on diversity collected by PTs (slope = 0.88, intercept = 1.52, p=0.07) (Figure 6C). At the 500 m radius scale, semi-natural habitat had no significant effect on wild pollinator abundance collected by FITs (slope = 0.72, intercept = 0.88, $p > 0.05$) or PTs (slope = 0.84, intercept = 1.36, $p > 0.05$) (Figure 6D). Semi-natural habitat had a significant positive impact on wild pollinator rarefied species richness collected by both FITs (slope = 7.31, intercept = 4.86, $p < 0.001$) and PTs (slope = 7.20,

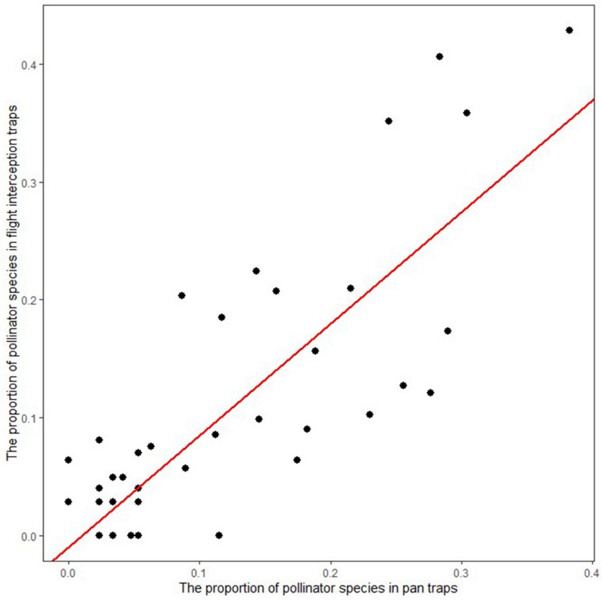

FIGURE 4
The relationship between the proportion of pollinator species in PTs and FITs.

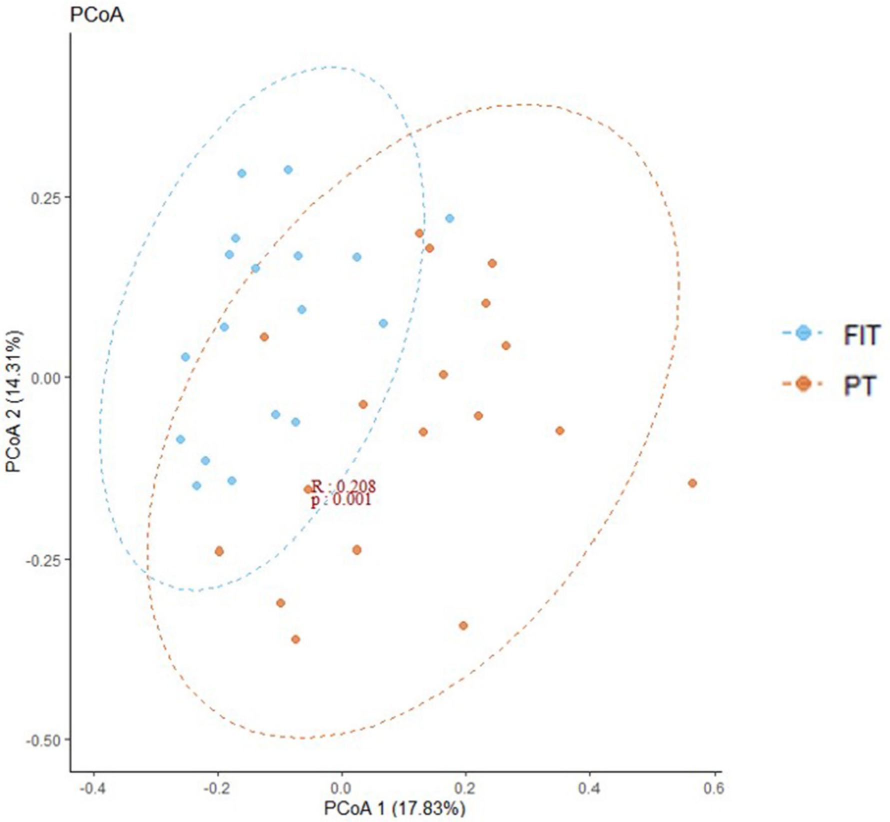

FIGURE 5
Principal coordinate analysis (PCoA) ordination of wild pollinator communities using pan traps (PT) and flight interception traps (FIT). The ellipses indicate 95% confidence intervals.

intercept = 6.13, $p < 0.05$) (Figure 6E). Semi-natural habitat in the agricultural landscape had significant positive impact on wild pollinator diversity collected by FITs (slope = 1.13, intercept = 1.31, $p < 0.001$) and PTs (slope = 0.82, intercept = 1.68, $p < 0.05$) (Figure 6F).

Discussion

Despite much prior research on the topic, there remain many gaps in our knowledge of effective monitoring for pollinating insects, especially in subtropical environments of Asia and for the multitude of passive trap methods (Cane et al., 2000; Campbell and Hanula, 2007; González et al., 2020; Van Drunen et al., 2022). The sampling performance of FITs and PTs, as two common passive pollinator sampling methods, has been assessed at limited scales (one oilseed rape field) previously and found flight interception trap outperform pan trap (more individuals and species collected per trap; Shi et al., 2022b). Here, we expanded on prior efforts by expanding sampling coverage and incorporating trait and semi-natural area analyses to determine whether these factors introduce biases.

Our results suggested that both FITs and PTs are surprisingly similar in their per-cost effectiveness and coverage in trapping pollinators. Results showed that a single FIT caught more individuals and species than single PTs, agreeing with the prior study (Rubene et al., 2015; Shi et al., 2022b). It seems likely that the highly attractive oilseed rape reduces the catches of PTs, so non-attractant FITs are less impacted by this and collect more specimens per unit (Baum and Wallen, 2011; Vrdoljak and Samways, 2012; Prendergast et al., 2020).

To scale for large monitoring efforts, cost must also be considered. We found that PTs (four per site) outperformed FITs (one per site, similar in cost to four PT sets) in oilseed rape fields (more wild pollinator individuals and species), while there was also now a difference in pollinator community composition between the two trap types. Notably, FITs can still manage to catch wild pollinator species that PTs did not, such as *Xylocopa nasalis* (a common carpenter bee species widely distributed in southern China), and more individuals of *Apis cerana*. In addition, FITs can be used to track wild pollinator movement patterns in agricultural landscapes (Cunningham et al., 2013) while PTs cannot. Thus, depending on the budget and research questions, FITs might still be useful under some circumstances.

More small pollinators were collected by pan traps and more small species, such as *Lasioglossum* sp, were significantly caught by pan traps, while there were no significant differences in the collection of large-bodied species between PTs and FITs, an outcome previously unreported. Contrastingly, McCravy et al. (2019) found that Malaise traps, which are somewhat similar to

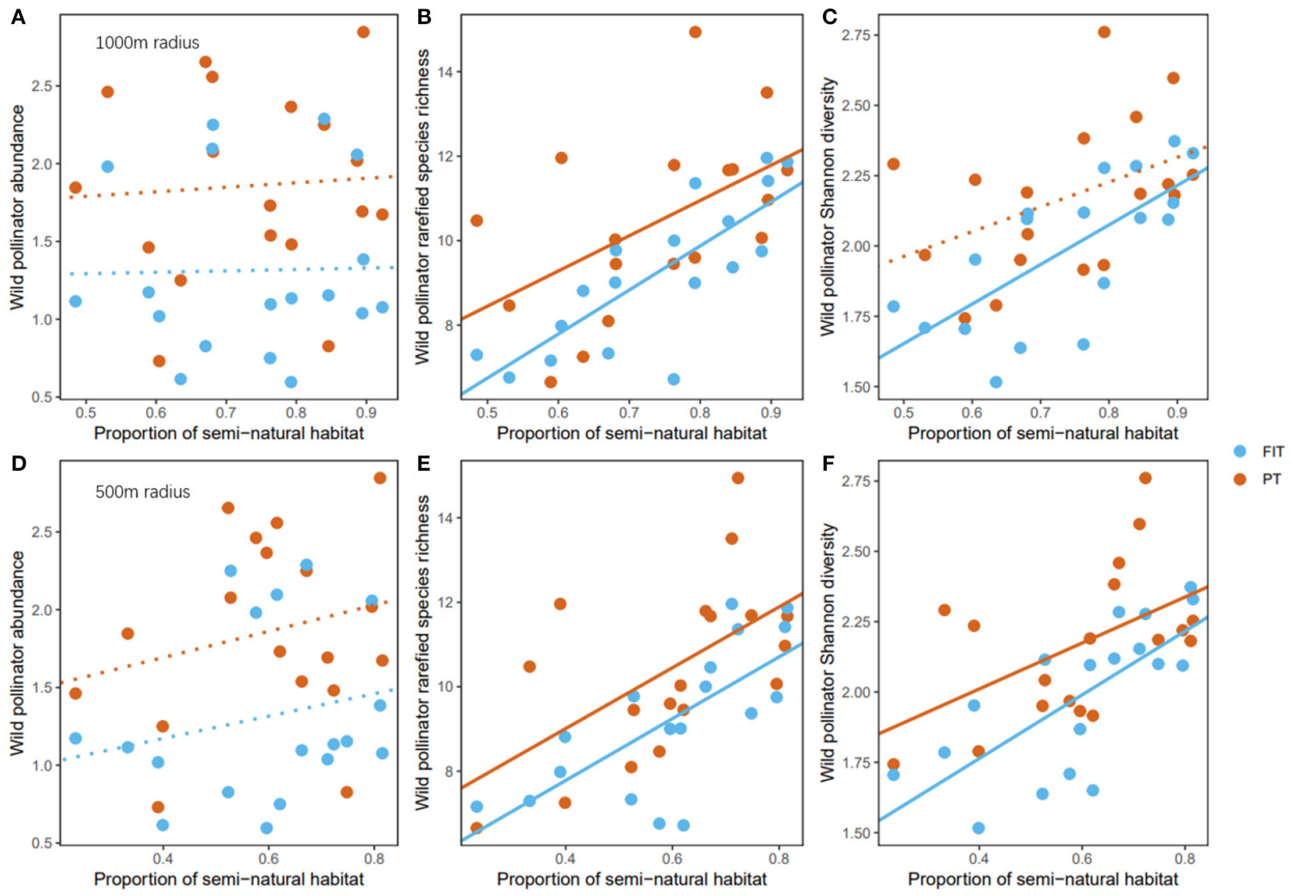

FIGURE 6
The impact of sampling methods (FIT and PT) and the proportion of semi-natural habitat in on **(A, D)** wild pollinator abundance (per sampling day), **(B, E)** rarefied species richness and **(C, F)** Shannon diversity. **(A–C)** were in 1000 m radius and **(D–F)** were in 500 m radius scale. Solid lines occur where the impact of semi-natural habitat was significant ($p < 0.05$).

TABLE 1 The impact of sampling methods (FIT and PT; base: FIT), the proportion of semi-natural habitat and their interactions on wild pollinator abundance per sampling day, rarefied species richness and Shannon diversity in large scale (1000 m radius) and small scale (500 m radius).

Explanatory variable	500 m			1000 m		
	Abundance	Rarefied species richness	Diversity	Abundance	Rarefied species richness	Diversity
Semi-natural habitat	0.72 ± 0.83	7.31 ± 2.20**	1.13 ± 0.31***	0.09 ± 1.09	10.43 ± 2.75***	1.41 ± 0.41**
Trap type (Base: FIT)	0.47 ± 0.73	1.27 ± 1.93	0.37 ± 0.27	0.40 ± 1.15	2.75 ± 2.92	0.57 ± 0.43
Semi-natural habitat*Trap type	0.12 ± 1.18	−0.11 ± 3.11	−0.32 ± 0.44	0.20 ± 1.54	−2.09 ± 3.89	−0.53 ± 0.58

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

FITs in that they physically block flight for capture, collected a higher proportion of small wild bees than PTs and vane traps. Krahner et al. (2021) also found that Malaise traps collected higher proportion of small wild bee than PTs, but their results were not consistent across 2 years. Consequently, it seems that these methods may both be better than malaise traps for sampling larger species, but this requires further study, and the unique collection of *Xylocopa nasalis* by only FITs suggests that perhaps some of the largest pollinators might still more easily avoid PTs.

Semi-natural habitat also had consistent positive impact on wild pollinator diversity and species richness across trap types. Semi-natural habitat inside agricultural landscapes can benefit wild pollinators through providing diverse floral resources as food, potential nesting sites and nesting materials (Tscharntke et al., 2005), and numerous studies have confirmed the important role of semi-natural habitats in maintaining wild pollinator diversity (Le Féon et al., 2010; Carvell et al., 2011; Zou et al., 2017; Wu et al., 2019; Shi et al., 2021). Prior studies have suggested that oilseed rape, as high-quality pollen and nectar source, can be extremely attractive for pollinators (Holzschuh et al., 2013) but both trapping methods recovered a positive effect of semi-natural areas nonetheless, possibly due to pesticides or other management regime factors specific to the area.

Many ecological studies integrate multiple trap types. The most commonly used combination is active sweeping net and passive PTs (Morandin et al., 2007; Kwaiser and Hendrix, 2008; Gill and O'Neal, 2015; Perrot et al., 2018; Van Drunen et al., 2022), and it is generally accepted that such a mix of active and passive methods is optimal (Prendergast et al., 2020). Many such studies have pooled samples across methods (Morandin and Winston, 2005; Tucker and Rehan, 2018), but some studies choose to analyze the results from different approaches separately (Perrot et al., 2018). FITs and PTs have also previously been analyzed together (Kehinde and Samways, 2012; Rader et al., 2014), but the consistency of these two traps across habitat types was previously unknown. With further testing in additional sites, it may be that FITs and PTs can be generally analyzed together, such that multiple questions can be addressed. To avoid overgeneralization, more sampling projects ought to be conducted at other sites, such as Jiangxi, Fujian and Hunan provinces, to comprehensively understanding the pollinator community in smallholder farmland in China. In addition, the potential competition between different methods used at the same sampling point (i.e., one trap may affect the other trap's catches) should be explored. Other potential concerns, for instance, causing damaging to crop when deploying traps and collecting samples, should be addressed in further studies.

Conclusion

In conclusion, PTs were more efficient than FITs in oilseed rape fields across various agricultural landscapes with a gradient of semi-natural habitat coverage in a cost-effectiveness framework. In terms of monitoring large and small-bodied pollinators, PTs collected more small individuals while there were no significant differences between FITs and PTs. Both also produced consistent results when investigating the impact of semi-natural habitat on wild pollinator diversity and species richness (semi-natural habitat had a significant positive impact on wild pollinator diversity and rarefied species richness). Consequently, PTs appear to be a viable method for monitoring pollinator diversity in subtropical agroecosystems, and these efforts could be enhanced by incorporating active sampling methods such as netting (Prendergast et al., 2020). Notably, although we recovered similar catches from these two methods, their sampling efficiency may vary in different habitats. Thus, further testing in additional habitats and climate would be useful for developing a generalizable guide for what methods should be used under different circumstances. Given the predominance of PTs in the literature, we suggest that for comparability purposes they should be preferred when limited resources for passive trapping are available, with resources devoted additionally to active methods that would better complement passive methods to provide a clearer view of the fauna.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

X-YS, C-DZ, and YZ conceived the study. MO, HQ, AL, Q-SZ, PG, M-QW, and ZN were involved in the study design. X-YS collected the data and conducted the statistical analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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EDITED BY
Fang Ouyang,
Institute of Zoology (CAS), China

REVIEWED BY
Fajun Chen,
Nanjing Agricultural University, China
Xiaobin Shi,
Hunan Academy of Agricultural Sciences, China

*CORRESPONDENCE
Xingyuan Men
✉ menxy2000n@hotmail.com

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Behavioral mechanism of transfer and dispersal of *Propylaea japonica* in cotton adjacent to sorghum fields

Hongying Cui, Lili Li, Yingying Song, Wenxiu Guo, Suhong Lv, Yi Yu and Xingyuan Men*

Institute of Plant Protection, Shandong Academy of Agricultural Sciences, Jinan, China

Increasing crop biodiversity, such as by adjacent managed crops, is recognized as an effective biological control measure. However, few studies have focused on the mechanisms involved in how adjacent managed crops increase natural enemy populations, leading to reduced pest numbers. This study investigated the hypothesis that cotton grown adjacent to sorghum would positively influence the feeding and oviposition preferences of the ladybug *Propylaea japonica*, which predates cotton aphids, leading to enhanced pest control. The populations of *Aphis gossypii* were significantly lower and those of *P. japonica* were significantly higher in cotton grown adjacent to sorghum compared with monoculture cotton fields. Regardless of diet on which the larva of *P. japonica* were reared (*Melanaphis sacchari*, *A. gossypii*, and 50% *M. sacchari* + 50% *A. gossypii*), the adults always consumed significantly more *M. sacchari* compared with *A. gossypii. P. japonica* also showed significantly higher feeding and oviposition preferences for host plants bearing aphids to only host plants. *P. japonica* fed *M. sacchari* preferred to lay eggs on cotton, whereas those fed *A. gossypii* preferred to lay eggs on sorghum. These results suggest that the habitat of natural enemies can be expanded by influencing their feeding and oviposition preferences to achieve pest control in adjacent cropping systems. This research, which incorporates field and laboratory studies, suggests an approach for the successful conservation and biological control of cotton aphids using adjacent managed cotton and sorghum crops.

KEYWORDS

adjacent managed, *Propylaea japonica*, feeding preference, oviposition preference, *Aphis gossypii*, *Melanaphis sacchari*

Introduction

Large monoculture farming systems reduce farmland biodiversity and have altered the composition and stability of arthropod populations in agriculture, promoting pest outbreaks as well as decreasing the number and effectiveness of natural enemies, particularly indigenous generalist predators (Thies et al., 2005; Mkenda et al., 2019). The adjacent management of crops has been shown to be an efficient tool to enhance the abundance and diversity of natural enemies and reduce the abundance of pests, decreasing crop damage and providing direct benefits by reducing the need for pesticides (Meehan et al., 2011; Paredes et al., 2013). High crop species richness can suppress pest populations, suggesting that crop species richness also enhances biological control services (Sheng et al., 2017). Greenstone et al. (2014) reported that growing soybean adjacent to cotton as part of a conservation biological control strategy significantly decreased the number of the target pest, *Megacopta cribraria* shield bugs. Natural enemies that moved from adjacent wheat fields to cotton fields were able to maintain the cotton aphid population below the threshold at which significant damage would be caused (Men et al., 2004). Therefore, adjacent crop management has been used to increase natural enemy efficiency to reduce the incidence of crop pests (Scheid et al., 2011; Simpson et al., 2011). There have been many field studies of adjacent crop management on the interactions of pests and their natural enemies. However, little is known about the mechanisms involved.

Host plants on which prey feed affect the nutritional quality of those prey, in turn affecting the feeding preference, development, and mortality of their predators (Banihashemi et al., 2017). The nutritional quality of host plants is an important factor influencing the vigor of predators because not all prey species are equally nutritious (Zhang et al., 2012). Plants respond to herbivore-induced damage by releasing density-related volatiles (Cotes et al., 2015). These volatiles can be used by natural enemies to find their prey on infested plants (Pettersson et al., 2005). For example, the adults of many natural enemies of aphids orient to volatile compounds emitted by host plants in response to aphid feeding (Sasso et al., 2009). Volatiles from aphid-infested cotton have a key role in mediating the orientation behavior of the ladybug *Propylaea japonica* (Thunberg) (Coleoptera: Coccinellidae) (Wang et al., 2015). Such attraction is likely to increase the fitness of *P. japonica* because aphids represent a complete food source for both adults and juveniles (Obrycki et al., 2009).

Both the presence and quality of prey have strong effects on not only the retention of adult predators, but also their reproductive output (Hodek and Honek, 2009). For example, the quantity or quality of a prey directly influence whether a female predator will oviposit on the host plant (Yao et al., 2021). In general, natural selection favors predators that lay eggs in a site that is most appropriate for their offspring (Putra et al., 2009). For example, egg clusters of the ladybug *Hippodamia convergens* are typically found only on aphid-infested sorghum plants in the field but not on uninfested plants (Michaud and Jyoti, 2007). In addition, the abundance and quality of aphids in a habitat affects the survival of larvae (Seagraves, 2009). There is an optimal number of coccinellid eggs that can be laid in an aphid colony to maximize the number of surviving offspring (Rondoni et al., 2014) and aphids constitute a staple food for ladybugs that oviposit in the vicinity of aphid colonies (Oliver et al., 2006). However, how predator feeding and oviposition preferences suppress aphids in adjacent managed host crops is unclear.

Aphids are very serious insect pests in most agroecosystems in the world (Figueroa et al., 2018). The cotton aphid *Aphis gossypii* and sugarcane aphid *Melanaphis sacchari* are the two most dominant species in northern China (Ma et al., 2006; Guo et al., 2011). *A. gossypii* is an important cotton pest, which causes severe damage to crops, leading to economic losses, whereas *M. sacchari* is one of the most important pests on sorghum (Wu and Guo, 2005; Guo et al., 2011). The ladybird *P. japonica* preys upon a variety of crop pests in northern China, predominantly aphids and, thus, serves as an excellent biological control agent (Gao et al., 2010). Aphid availability and quality affect the fecundity and survival of *P. japonica* (Tang et al., 2013). Previous studies showed that sorghum was a source of ladybirds in cotton and, thus, incorporation of sorghum on farms growing cotton has the potential to enhance the biocontrol of cotton aphids on cotton in the field (Tillman and Cottrell, 2012). However, the mechanisms affecting the transfer and dispersal of *P. japonica* between sorghum and cotton remain to be elucidated.

Thus, the current study investigated: (1) the effects of adjacent managed cotton–sorghum ecosystems on *P. japonica* and its aphid prey; (2) the effects of aphid species on the consumption by, and behavioral responses of, *P. japonica* fed *M. sacchari*, *A. gossypii*, and 50% *M. sacchari* + 50% *A. gossypii*; and (3) the effects of host plant type (sorghum; cotton; sorghum inhabited by *M. sacchari*; and cotton inhabited by *A. gossypii*) on the feeding and oviposition preferences of adult *P. japonica*.

Materials and methods

Field experimental design

The field experiment was performed in 2021 at the Experimental Base of Shandong Agricultural Academy of Science, Jinan, Shandong, China (116.99°E, 36.97°N). Experimental units were 10 m × 100 m sorghum plots (variety Lunuo 8) planted adjacent to 40 × 100 m cotton plots (variety Lumianyan 28). A mono cotton field was used as a control. In the cotton and sorghum fields, each plot was sampled by using a 5-point random sampling method, and the number of *A. gossypii*, *M. sacchari*, and *P. japonica* on three cotton and three sorghum plants per point was recorded on 16 August 2021.

P. japonica breeding

Individuals *P. japonica* were collected from the Experimental Base of Shandong Agricultural Academy of Science in the field. The offspring of these *P. japonica* were reared in an artificial chamber (PRX-500D-30, Haishu Safe Apparatus, Ningbo, China), which was maintained at 28°C and 75% relative humidity (RH) under a photoperiod of 14 h:10 h light:dark. Newly hatched *P. japonica* were fed on three different diets (*M. sacchari*, *A. gossypii*, or 50% *M. sacchari* + 50% *A. gossypii*).

Host plants

Plants of two crops, cotton (variety LuMianYan 28) and sorghum (variety Lunuo 8), were selected for this study. Plants were grown in a potting mixture of peat moss, vermiculite, organic fertilizer, and perlite (10:10:10:1 by volume) in a greenhouse under natural light at 28 ± 2°C. Plants were randomly placed in the artificial chamber and re-randomized once a week to avoid positioning effects. No fertilizer or pesticides were used throughout the experiment.

Feeding and oviposition preference of *P. japonica*

The feeding preference of *P. japonica* was evaluated on sorghum, cotton, sorghum inhabited by *M. sacchari*, and cotton inhabited by *A. gossypii*. *P. japonica* individuals were collected directly from fields located in the Experimental Base, Shandong Agricultural Academy of Science. The experiment was conducted in a covered square cage (2.0 × 2.0 × 2.0 m). Twelve plants (three sorghum plants, three cotton plants, three sorghum plants inhabited by *M. sacchari*, and three cotton plants inhabited by *A. gossypii*) were placed in a random order at an equal distance from the center point to form a circle. Twenty adults *P. japonica* were placed on the center point within a replicate. The positions of the adult beetle were checked and recorded daily at 2-h intervals from 05:00 h to 21:00 h for 3 days.

The oviposition preference of *P. japonica* was assessed following the same method as described above for the feeding preference. *P. japonica* fed on *M. sacchari*, *A. gossypii*, and 50% *M. sacchari* + 50% *A. gossypii,* respectively. Five mated and ovipositing adult females (10 days old) were released in the center of square cover cage. The number of eggs laid on each plant was counted after 3 days to determine the oviposition preference.

Prey biomass consumption

The prey biomass consumed by *P. japonica* was determined using no-choice and free choice tests. A 24-h-starved adult female *P. japonica* (10 days old) that had previously been fed one of three different diets (*M. sacchari*, *A. gossypii*, or 50% *M. sacchari* + 50% *A. gossypii*) was provided with third-instar larvae of *M. sacchari* and *A. gossypii*. In the no-choice test, *P. japonica* was provided with 100 *M. sacchari* or *A. gossypii* larvae. In the free choice test, *P. japonica* was provided with 50 *M. sacchari* and 50 *A. gossypii* larvae. After 24 h, the number of unconsumed aphids was counted. The biomass consumption of *P. japonica* was then calculated based on the weight of 100 aphids and feeding ratio.

Y-tube experiments

A Y-tube olfactometer was used to investigate the behavioral responses of *P. japonica* adults that had fed on one of five diets (*M. sacchari*, *A. gossypii*, or 50% *M. sacchari* + 50% *A. gossypii*, fed on *M. sacchari* then fed on *A. gossypii* for 1 day and fed on *A. gossypii* then fed on *M. sacchari* for 1 day) to: (1) intact sorghum vs. intact cotton; (2) sorghum bearing *M. sacchari* vs. cotton bearing *A. gossypii*; and (3) *M. sacchari* vs. *A. gossypii*. For the treatments of different plants, plant was placed in a glass enclosure which connected to the ends of arms with five centimeters rubber tube. For the treatment of *M. sacchari* vs. *A. gossypii*, aphids were placed on the end of the arms.

The behavioral responses of *P. japonica* were determined in a 40 mm-diameter × 36 cm-long glass Y-tube olfactometer with a 60° inside angle. The flow rate was 4.8 L/min (equal to 3.8 m/min inside the tube) in each Y-tube arm. A single *P. japonica* was placed in the olfactometer for 10 min. A "no choice" outcome was recorded when the adults remained inactive during the test period. A "first choice" outcome was recorded when the adults moved >25 cm into either arm (visually assessed by a line marked on each arm). Each experimental pair was repeated with at least adult 80 *P. japonica*.

Statistical analysis

Two-way factorial ANOVA (SPSS 13.0, SPSS Inc., Chicago, IL, USA) was used to analyze the feeding preference and prey biomass consumption of adult *P. japonica*. One-way ANOVA was used to analyze the effect of adjacent crop management on the population numbers of *M. sacchari*, *A. gossypii*, and *P. japonica* in the field. Differences among means were determined using Tukey's test at $P < 0.05$. X^2 tests were used to analyze the adult *P. japonica* feeding and oviposition preferences and to examine the significance of differences in the choosing behaviors of *P. japonica* in the olfactometer test.

Results

Effect of adjacent crop management on the numbers of *M. sacchari*, *A. gossypii,* and *P. japonica* in the field

The number of *M. sacchari* on sorghum was significantly higher than the number of *A. gossypii* on adjacent cotton (89.68%; $F = 21.32$, $P < 0.001$; Figure 1A). The same was found for *A. gossypii* populations on mono cotton compared with the number on adjacent cotton (67.05%; $F = 35.90$, $P < 0.001$; Figure 1B). Similarly, the number of *P. japonica* per 100 sorghum plants was significantly higher than on adjacent cotton (84.73%; $F = 209.05$, $P < 0.001$; Figure 1C), as was the number of *P. japonica* per 100 adjacent cotton plants compared with mono cotton ($F = 18.48$, $P < 0.001$; Figure 1D).

Feeding preference

Host plant type significantly influenced the feeding preference of adult *P. japonica*, whereas time of day and host plant type × time of day did not (Table 1). *P. japonica* preferred sorghum to cotton, and with a significantly higher feeding preference for both types of host plant bearing aphids compared with host plants without aphids (Figure 2).

Y-tube experiments

P. japonica fed *M. sacchari* showed a significant preference for the odor of sorghum compared with cotton ($P < 0.01$; Figure 3A). However, there was no difference in preference for host plant odor between the other four treatment groups (i.e., *P. japonica* fed 50% *M. sacchari* + 50% *A. gossypii*, *A. gossypii* + *M. sacchari* for 1 day, or *A. gossypii*, *M. sacchari* + *A. gossypii* for 1 day) (Figure 3A).

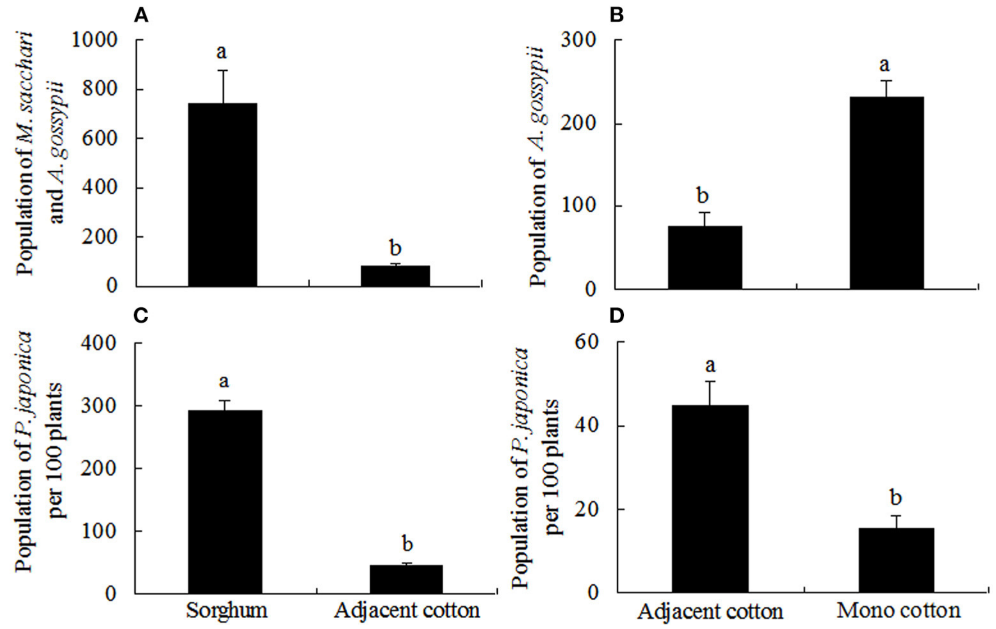

FIGURE 1
Effect of adjacent crop management on the numbers of *M. Sacchari*, *A. Gossypii* and *P. Japonica* in the field. **(A)** Population of *Melanaphis sacchari* on sorghum and *Aphis gossypii* on adjacent cotton. **(B)** Population of *A. gossypii* on adjacent cotton and mono cotton. **(C)** Population of *P. japonica* per 100 plants of sorghum and adjacent cotton. **(D)** Population of *P. japonica* per 100 plants on adjacent cotton and mono cotton. Different lowercase letters indicate significant differences among different crops (Tukey's test: $P < 0.05$).

TABLE 1 ANOVA of the effects of host plant type and time of day on the feeding preference of *Propylaea japonica*.

Source	df	*F*	*P*
Host plant type	3	269.223	<0.001
Time of day	5	0.026	1
Host plant type × time of day	15	0.974	0.488

P. japonica fed *M. sacchari* showed a significant preference for the odor of sorghum bearing *M. sacchari* compared with cotton bearing *A. gossypii* ($P < 0.05$; Figure 3B). However, there was no difference in preference for sorghum bearing *M. sacchari* vs. cotton bearing *A. gossypii* between the other four treatment groups (see above) (Figure 3B).

P. japonica fed *M. sacchari* significantly preferred *M. sacchari* to *A. gossypii* ($P < 0.001$; Figure 3C). However, there was no difference in preference for *M. sacchari* vs. *A. gossypii* in any of the other four treatment groups (see above) (Figure 3C).

Prey biomass consumption

Aphid species significantly influenced the biomass consumed by *P. japonica* in the no-choice and free choice tests (Table 2).

In the no-choice test, *P. japonica* adults fed *M. sacchari* ($F = 5.12$, $P < 0.05$), *A. gossypii* ($F = 4.72$, $P < 0.05$), and 50% *M. sacchari* + 50% *A. gossypii* ($F = 6.50$, $P < 0.05$) consumed significantly more *M. sacchari* compared with *A. gossypii* (by 25.74, 21.02, and 24.07%, respectively) (Figure 4A). However, there was no significant difference in the biomass of *M. sacchari* or *A. gossypii* consumed by *P. japonica* adults fed on above three treatments (Figure 4A).

In the free choice test, *P. japonica* adults fed *A. gossypii* ($F = 5.61$, $P < 0.05$) and 50% *M. sacchari* + 50% *A. gossypii* ($F = 26.27$, $P < 0.001$) consumed significantly more *M. sacchari* compared with *A. gossypii* (30.83 and 45.68%, respectively) (Figure 4B). However, there was no significant difference in the biomass of *M. sacchari* or *A. gossypii* consumed by *P. japonica* adults fed on above three treatments (Figure 4B).

Oviposition preference

P. japonica fed *M. sacchari* significantly preferred to lay their eggs on cotton compared with sorghum, and on sorghum bearing *M. sacchari* rather than sorghum alone ($X^2 = 7.630$, $P < 0.05$; Figure 5). *P. japonica* fed *A. gossypii* significantly preferred to lay their eggs on sorghum bearing *M. sacchari* compared with cotton bearing *A. gossypii*, and on sorghum bearing *M. sacchari* compared with sorghum alone ($X^2 = 5.678$, $P < 0.05$; Figure 5). *P. japonica* fed 50% *M. sacchari* + 50% *A. gossypii* significantly preferred to lay their eggs on host plants infested with aphids regardless of the aphid species compared with host plants only (cotton and sorghum) ($X^2 = 10.020$, $P < 0.05$; Figure 5).

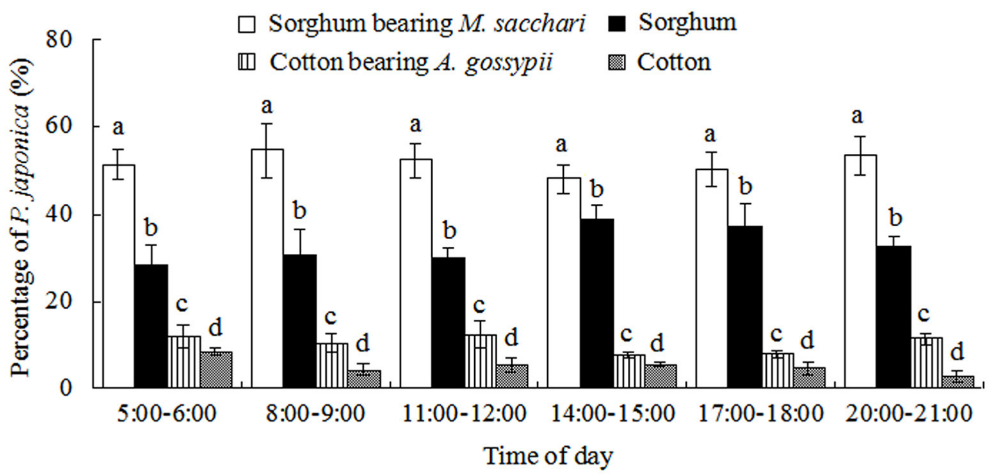

FIGURE 2
Effects of host plant type on the feeding preference of adult *Propylaea japonica*. Different lowercase letters indicate significant differences between host plant type treatments at a set time (X^2 test, $P < 0.05$).

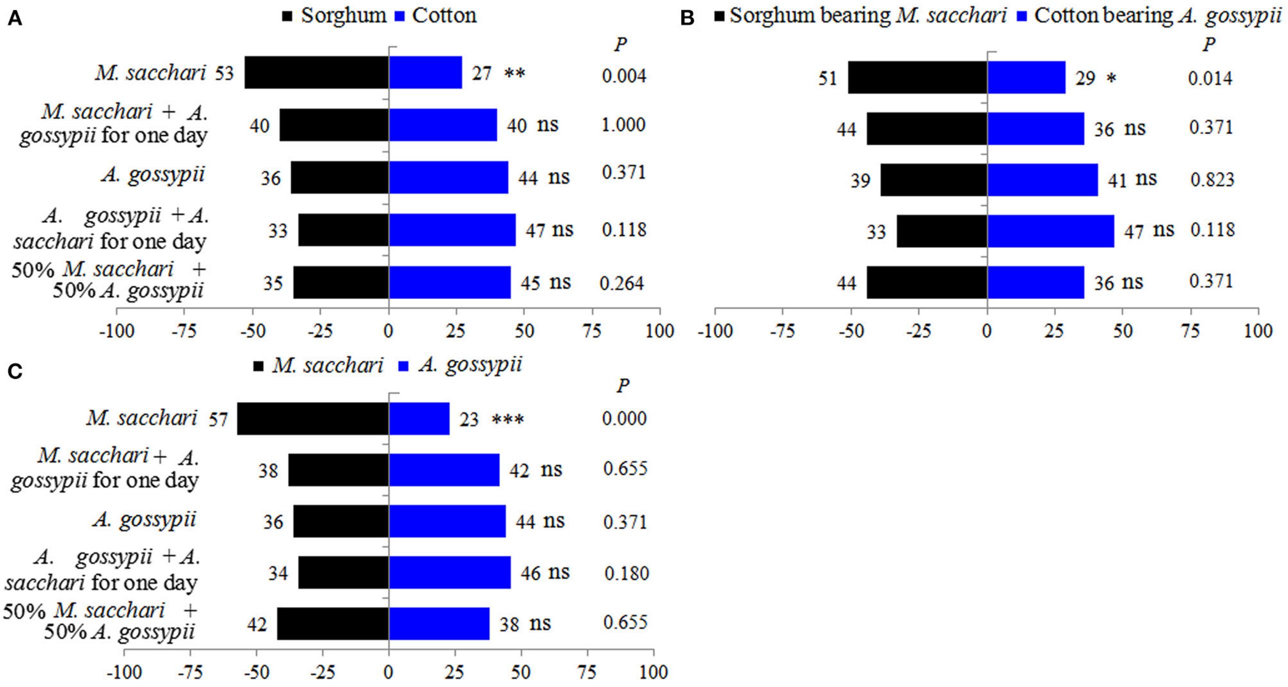

FIGURE 3
Effect of aphid species on the behavioral responses of *Propylaea japonica*, originally fed different diets (*M. sacchari*, *A. gossypii*, 50% *M. sacchari* + 50% *A. gossypii*, fed on *M. sacchari* then fed on *A. gossypii* for 1 day, or fed on *A. gossypii* then fed on *M. sacchari* for 1 day), to sorghum and cotton **(A)**, sorghum with *M. sacchari* and cotton with *A. gossypii* **(B)**, and *M. sacchari* and *A. gossypii* **(C)**. The X^2 test was used to analyze differences between the numbers of *P. japonica* in each arm of the Y-tube (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non-significant).

Discussion

Researchers have evaluated the role of crop diversity in improving the impact of biological control, which enhances natural enemies and reduces pests (Rusch et al., 2016). High crop species richness suppresses pest populations by increasing the number of natural enemies (Sheng et al., 2017). There is a growing body of evidence suggesting that adjacent habitats positively affect pest regulation by natural enemies compared with monoculture systems (Bianchi et al., 2010). Large numbers of natural enemy taxa that move to adjacent crops provide a biological pest control service (Macfadyen and Muller, 2013). For example, alfalfa grown adjacent to wheat fields significantly increased the abundance of predators, including *Hippodamia variegata* and *Chrysopa sinica*, and decreased the densities of *Macrosiphum avenae* and *Schizaphis graminum*, which are dominant cereal aphid species in wheat fields

(Zhao et al., 2013). In the current study, the number of *M. sacchari* on sorghum was significantly higher than of *A. gossypii* on adjacent cotton, whereas *P. japonica* populations were significantly higher on sorghum than on adjacent cotton, suggesting that the predator population increased with the increasing pest population on the host crop. In addition, *A. gossypii* populations were significantly smaller and *P. japonica* populations were significantly larger in adjacent cotton fields than in monoculture cotton fields. These results were consistent with previous studies of cotton grown adjacent to sorghum (Tillman and Cottrell, 2012). This suggests sorghum is an ideal crop for the conservation of predators that subsequently disperse to adjacent cotton fields and help control the aphid population.

The effects of a host plant on the palatability and suitability of the prey for a predator are well-known (Ugine et al., 2021). Prey preference and prey suitability are important for determining predator behavior, such as feeding and habitat selection (Weber et al., 2006). Published data indicate that induced volatiles attract natural enemies to an infested plant (Tan and Liu, 2014). For example, *Harmonia axyridis* females showed a significant preference for aphid-infested marigolds because of the plant volatiles induced by aphid feeding (Zhang et al., 2022). In the current study, *P. japonica* showed a significantly higher feeding preference for host plants bearing aphids compared with host plants alone. This might be because aphid-infested host plants, such as sorghum and cotton, release significantly higher amounts of volatiles compared with host plants without aphids, thus attracting higher numbers of natural enemies. Our results also showed that *P. japonica* preferred sorghum to cotton, which might be because sorghum is a C4 plant, whereas cotton is a C3 plant. Thus, crops grown adjacent to C3 (cotton) or C4 (sorghum) plants provide ideal systems for studying the transfer and dispersal of natural enemies.

Differential biomass consumption is indicative of aphid palatability to ladybugs (Mishra, 2005). Previous studies showed *M. sacchari* is an optimal food source for *P. japonica* among five species of aphids (Liu et al., 2013). The current results showed that *P. japonica* adults consumed significantly more *M. sacchari* compared with *A. gossypii.* This suggests that *P. japonica* prefers to consume *M. sacchari*, which could explain the increased *P. japonica* populations found in sorghum crops grown adjacent to cotton in the field. In our study, *P. japonica* fed *M. sacchari* also showed a significant preference for the odor of sorghum, sorghum bearing *M. sacchari*, and *M. sacchari* alone. After being fed *A. gossypii* for 1 day, these preferences disappeared. This suggests that, as *M. sacchari* populations decrease in the field, *P. japonica* would move to feed on *A. gossypii*, which could lead to a change in preference of *P. japonica* for *M. sacchari*, resulting in the transfer and dispersal of *P. japonica* in adjacent managed cotton–sorghum ecosystems.

The type of plant can also affect its suitability as a oviposition site for predators, such as ladybugs, and subsequently affect their reproduction (Mirhosseini et al., 2015). Host plants can have a dramatic effect on the survival of coccinellid eggs, with *Coleomegilla maculata* being shown to preferentially lay eggs on aphid-infested plants (Michaud and Jyoti, 2007). Female *Propylea*

TABLE 2 ANOVA of the effects of *P. japonica* treatment (fed on different diets) and aphid species on the aphid biomass consumed by *Propylaea japonica*.

Different treatment	Factor	*F*	*P*
No choice test	*P. japonica* treatment	1.774	0.178
	Aphid species	16.045	<0.001
	P. japonica treatment × aphid species	0.037	0.963
Free choice test	*P. japonica* treatment	1.126	0.332
	Aphid species	28.361	<0.001
	P. japonica treatment × aphid species	1.74	0.185

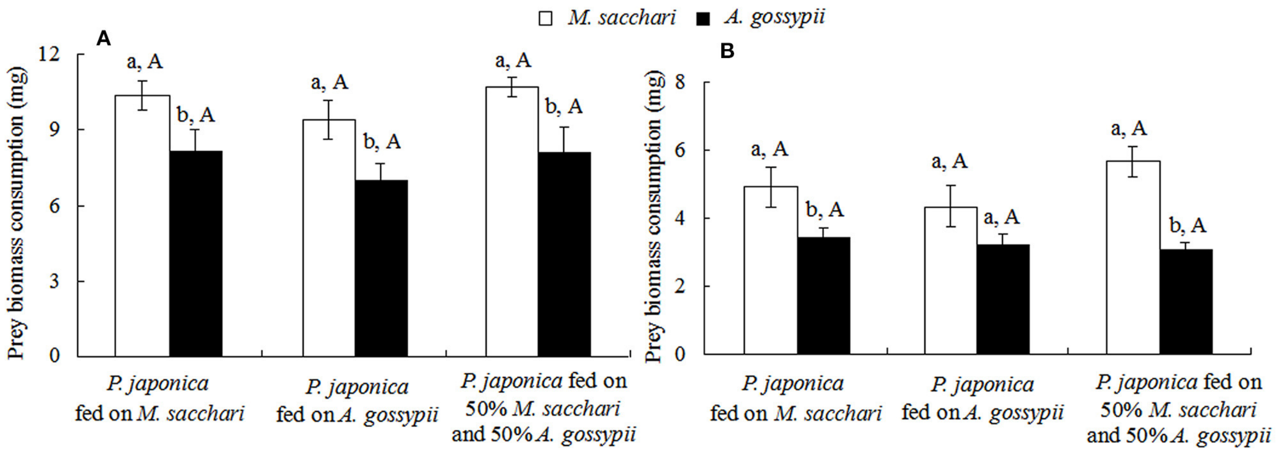

FIGURE 4
Effects of aphid species on the prey biomass consumed by adult *Propylaea japonica* fed different aphid-based diets (*M. sacchari*, *A. gossypii*, and 50% *M. sacchari* + 50% *A. gossypii*) in the no-choice test **(A)** and free-choice test **(B)**. Different lowercase letters indicate significant differences between aphid species within a *P. japonica* treatment, and different uppercase letters indicate significant differences between *P. japonica* treatments involving different aphid species (Tukey's test: $P < 0.05$).

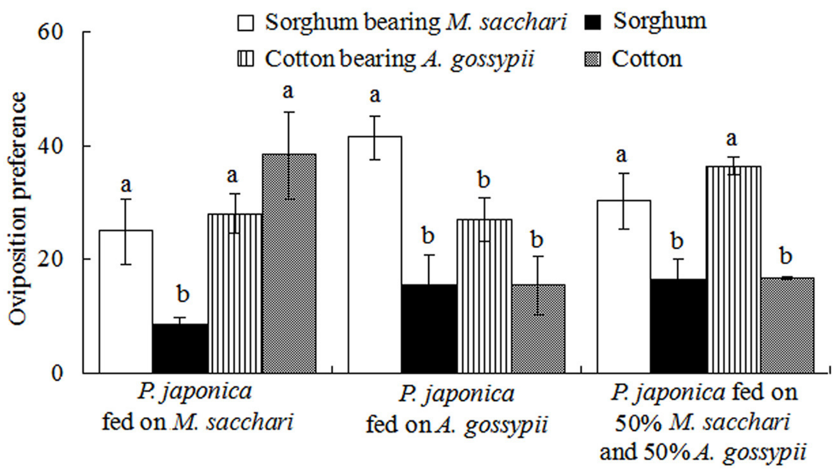

FIGURE 5
Effect of host plant on the oviposition preference of adult *Propylaea japonica* originally fed different aphid-based diets (*M. sacchari*, *A. gossypii*, and 50% *M. sacchari* + 50% *A. gossypii*). Different lowercase letters indicate significant differences between the type of host plant treatment within a *P. japonica* treatment (X^2 test: $P < 0.05$).

dissecta laid numerous eggs on plants with a high aphid density and fewer eggs on plants with a low aphid density (Omkar, 2004). In addition to coccinellids, oviposition preference of hoverflies varies in response to both the presence of aphids as well as their aphid (Amiri-Jami et al., 2016). In the current study, *P. japonica* significantly preferred to lay their eggs on aphid-infested cotton and sorghum, a result consistent with previous studies, presumably because the aphids are a food source for the larvae once they've hatched. It was also found that *P. japonica* fed *M. sacchari* preferred to lay eggs on cotton, whereas those fed *A. gossypii* preferred to lay eggs on sorghum. This suggests that oviposition behavior can be exploited to expand the habitat of ladybugs, an ecological adaptation of predatory natural enemies in the farmland ecosystems.

Conclusions

Our results indicate that an adjacent cropping system of cotton and sorghum can result in significantly higher *P. japonica* populations, leading to decreased aphid abundance compared with monocultures. *P. japonica* preferred to feed and lay eggs on aphid-infested plants over host plants without aphids, and also prefer to oviposit on other host plants. These results suggest that the habitat of natural enemies can be expanded by influencing their feeding and oviposition preferences to achieve pest control in adjacent cropping systems. Thus, increasing crop diversity, which provides favorable conditions for agriculture based on ecological principles, can contribute to the development of sustainable agroecosystems.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

HC and XM conceived, designed, and performed the experiments. LL, YS, WG, SL, and YY contributed reagents, materials, and analysis tools. HC analyzed the data and wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Javad Karimi,
Ferdowsi University of Mashhad, Iran

REVIEWED BY
Muhammad Hafeez,
Zhejiang University, China
Hana Haji Allahverdipour,
Iranian Research Institute of Plant Protection
(IRIPP), Iran

*CORRESPONDENCE
Wenxiu Guo
✉ wenxiu.guo@163.com
Yi Yu
✉ robertyuyi@163.com

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Curative efficacy of entomopathogenic nematodes against white grubs in honeysuckle fields

Xia Li[1,2,3], Xingyuan Men[1], Jianhua Wang[2,3], Suhong Lv[1], Lili Li[1], Hongying Cui[1], Yingying Song[1], Xinsheng Fang[2,3], Zhenqiao Song[2,3], Wenxiu Guo[1]* and Yi Yu[1]*

[1]Shandong Key Laboratory of Plant Virology, Institute of Plant Protection, Shandong Academy of Agricultural Sciences, Jinan, China, [2]State Key Laboratory of Crop Biology, College of Agronomy, Shandong Agricultural University, Tai'an, China, [3]Shandong Key Laboratory of Crop Biology, College of Agronomy, Shandong Agricultural University, Tai'an, China

Root-feeding white grubs are one of the most serious pests of honeysuckle trees (*Lonicera japonica*) in China, directly damaging their roots and facilitating infection by soil pathogens. Entomopathogenic nematodes (EPNs) are considered as potential control agents against soil-dwelling insect pests. This study aimed to identify effective EPN species against white grubs through bioassay and field experiments. Among the EPN species screened against *Holotrichia oblita* under laboratory conditions, *Steinernema feltiae* and *Heterorhabditis indica* had low virulence, while *S. longicaudum*, *S. glaseri*, and *H. bacteriophora* applied at a rate of 400 IJs/larva caused a higher corrected mortality (80.00 ± 5.77%), which screened them as good candidates for future applications. The field experiments showed that both *S. longicaudum* and *H. bacteriophora* were approximately as effective in reducing white grubs as the insecticide phoxim, whereas *S. glaseri* caused a significantly lower reduction compared with these two EPNs and phoxim. Plant mortalities obtained from *S. longicaudum*, *H. bacteriophora* and the insecticide treatment plots were significantly lower than those observed in the water-treated control plots. All EPNs examined could establish well in the treated honeysuckle fields for 42 d, confirmed by *Tenebrio molitar* larvae baiting technique. Our findings suggest that EPNs could provide curative efficacy against white grubs and significantly reduce plant death in honeysuckle fields.

KEYWORDS

white grubs, entomopathogenic nematode, honeysuckle, biological control, field efficacy, ecological planting

1. Introduction

Honeysuckle, *Lonicera japonica* Thunb, is a Chinese medicinal plant native to East Asia and can be easily grown all over the world. It is renowned for its active compounds and widespread pharmacological effects on heat-evil, dysentery and swellings, body protection and lifespan extension as recorded in the famous classical book of Chinese material media "Ben Cao Gang Mu" (Shang et al., 2011). So many beneficial effects including anti-viral (Ding et al., 2017), anti-bacterial (Rahman and Sun, 2009), anti-oxidant (Kong et al., 2017), anti-inflammatory (Tang et al., 2016), anti-diabetic (Han et al., 2015) and neuroprotective (Wang et al., 2014) have been demonstrated for this plant. Moreover, it is also used as cosmetics, food products, and healthy beverages worldwide (Wang, 2010; Fang et al., 2020). Along with the great changes in the environment, food consumption, and lifestyle observed in the modern society, honeysuckle is playing an increasingly important role in our daily life (Yang et al., 2018). Honeysuckle cultivation has been expanded as the demand increases (Hu et al., 2022), while the problem of white grubs is becoming more and more serious (Xin, 2017; Li, 2022).

White grubs, which are the root-feeding larvae of scarab beetles, are one of the most severe soil-dwelling pests and are increasingly damaging honeysuckle cultivation (Xu and Wei, 2021). *Holotrichia oblita* Faldermann is one of the dominant species found in honeysuckle fields and it generally co-occurs with other white grub species, such as *Brahmina faldermanni* Kraatz and *Maladera orientalis* Motschulsky (Li, 2022). These larvae feed on honeysuckle roots, facilitating their infection by other soil pathogens and subsequent decay (Gao et al., 2020). The damages caused to the roots affect the entire plant, with serious impacts on tree growth and flowering, eventually leading to the plant's death (Liu et al., 2017; Gao et al., 2020).

For many years, white grubs in honeysuckle fields have been mainly controlled using chemical insecticides, such as phoxim and chlorpyrifos (Liu et al., 2017). However, the efficacy of these products is not always satisfactory as white grubs live concealed in the soil and in addition to the development of insecticide resistance (Gao et al., 2020). Therefore, in light of the increasing environmental and human safety concerns, and of the importance of honeysuckle flowers for medical purposes, alternative biological strategies are urgently needed to control white grubs in honeysuckle fields.

Entomopathogenic nematodes (EPNs) are known as potential biological control agents and have been used to control a variety of soil-dwelling insects due to their superior ability to actively search for hosts (Grewal et al., 2005; Georgis et al., 2006). Some EPN species have been shown to be potentially highly efficient against different white grub species in turf grass or peanut fields, such as *Steinernema scarabaei*, *S. longicaudum*, *S. glaseri*, *Heterorhabditis bacteriophora*, and *H. zealandica* (Tamson and Alm, 1995; Koppenhöfer et al., 2000, 2002; Koppenhöfer and Fuzy, 2003a,b; Grewal et al., 2004; Du et al., 2009; Guo et al., 2015). However, knowledge of EPNs application to control white grubs in honeysuckle fields is still limited.

The successful application of EPNs strictly depends on environmental factors, such as soil texture, moisture, and temperature (Shapiro-Ilan et al., 2012a; Guo et al., 2015). Honeysuckle cultivation needs to pay more attention to the geo-herbalism (Zhang et al., 2003; Duan et al., 2019), for the soil characteristics is of great importance to the content of active compounds in honeysuckle flowers (Chen et al., 2021). Yimeng mountain area is the natural planting area for honeysuckle (Liu et al., 2008). Pingyi county, which is located in the Yimeng Mountains, is the largest honeysuckle production area in China (Zhang, 2021). The soil here is sandy and arid, which favors the accumulation of the plants' active compounds (Chen et al., 2021). Whether these soil conditions are also suitable for the successful application of EPNs needs to be explored.

More importantly, it is necessary to choose the appropriate EPN species to control the target pest by considering their virulence, environmental tolerance, and even persistence (Shapiro-Ilan et al., 2002, 2006a,b). The virulence of EPNs to white grubs varies with EPN and white grub species (Koppenhöfer and Fuzy, 2003a,b; Grewal et al., 2004). Although there are some differences in the virulence of each EPN to different white grub species, certain EPNs were shown to be pathogenic to several white grubs, as observed for *H. bacteriophora* against *Popilia japonica* Newman (Selvan et al., 1994), *Maladera matrida* Argaman (Glazer and Gol'Berg, 1993), *H. parallela* Motschulsky (Guo et al., 2013), and *H. oblita* (Guo et al., 2015). Little is known on the efficacy of certain EPNs against white grubs in the honeysuckle fields. More EPN species are needed to be screened for providing more alternatives to effectively control white grubs that always co-occur in the same honeysuckle fields.

Therefore, five EPN species, i.e., *S. longicaudum* X-7, *S. glaseri* KG, *S. feltiae* SN, *H. bacteriophora* H06, and *H. indica* LN2, reported with high virulence or good performance in the fields against several pests, for example, fungus gnats, Lepidopterous pests and white grubs (Yan et al., 2014; Wang et al., 2021), were chosen for bioassay screening against *H. oblita*, one of the dominant white grub species in honeysuckle fields. Subsequently, the control efficacy of high virulent EPN species screened in the bioassay was evaluated in the honeysuckle fields in the present study.

2. Materials and methods

2.1. EPNs

The *Steinernema longicaudum* X-7, *S. glaseri* KG, *S. feltiae* SN, *H. bacteriophora* H06, and *H. indica* LN2 species used in this study were provided by Weifang Hongrun Agriculture Science and Technology Co., LTD, China. Infective juveniles (IJs) were cultured *in vitro* in solid sponge media using the method described in Bedding (1981) with modifications (Han, 1996) and were formulated with vermiculite (200 mesh). IJ suspensions were used for experiments if more than 95% of IJs were alive, which was assessed using a microscope before the experiments (Yan et al., 2013).

2.2. Insects

The second instar larvae of *H. oblita* used for the bioassays were provided by the Cangzhou Academy of Agriculture and Forestry Science, China. The white grubs were reared and fed on dry potato pieces (0.5 × 0.5 × 0.5 cm). The size and weight of each instar larva were consistent with the measurements reported in Guo et al. (2015). The larvae were individually kept in plastic cups (with a diameter of 4.3, height of 7 cm, and a 2-mm-diameter hole in the lid) filled with 50 g of sandy soil (10% w/w soil moisture) at 25 ± 2°C and 50% relative humidity (RH). Six wheat seeds were added to each cup as food. The cups were kept at 25°C for 24 h and only grubs that showed signs of activity were selected for the bioassays (Koppenhöfer and Fuzy, 2008).

Yellow mealworms, *Tenebrio molitor* L., were purchased from Shandong Taian Wuma market. The mealworms were reared in a controlled room with 25 ± 2°C and 50% RH, and fed on wheat bran and fresh vegetable leaves. Similarly sized 9th- to 11th-instar larvae were chosen to evaluate nematode persistence by burying them in soil samples collected from the field experiments (Guo et al., 2015).

2.3. Bioassays

2.3.1. Virulence of different EPN species to second instar *H. oblita* larvae

One mL of IJ suspensions containing 200 IJs of *S. longicaudum*, *S. glaseri*, *S. feltiae*, *H. bacteriophora*, or *H. indica* was applied into each cup containing the *H. oblita* larvae (equal to 1.5×10^9 IJs ha^{-1}). A similar volume of water without nematodes was added to the soil of control treatment. Three replicates were set for each treatment or control and each replicate tested 10 individual larvae. After the treatment, the cups were placed in the dark at 25 ± 2°C and 75 ± 5% RH. White grub mortality was assessed after 4, 7, and 14 d; the cadavers were placed onto moist filter paper and were dissected 3 d later to evaluate IJ invasion (Yan et al., 2013).

2.3.2. Effect of highly virulent EPN species applied at different rates

The bioassay was performed in plastic cups, as described above. IJ suspensions of the most effective EPN species screened in the first step, i.e., *S. longicaudum*, *S. glaseri*, or *H. bacteriophora*, were prepared. One mL of the nematode suspension containing 400, 200, 100, or 50 IJs was applied into each cup with one grub (equal to 3.0×10^9, 1.5×10^9, 7.5×10^8 or 3.75×10^8 IJs ha^{-1}). Water without IJs was used as control. Ten cups with 10 individual larvae were set as one replicate and three replicates were set for each treatment or control. All cups were placed in the dark at 25 ± 2°C and 75 ± 5% RH. Grub mortality was assessed as described in the previous section.

2.3.3. Field experiments

Two field trials were conducted in different honeysuckle fields in the Pingyi area, China. Before the treatments, the presence of native EPN populations in the fields was assessed by baiting soil samples with yellow mealworms as described in Liu et al. (2009). No EPN populations were detected in the experimental fields. Grub population was estimated based on Du et al. (2009) and species were identified following the guidelines reported in Wei et al. (1989) and Cao and Li (2017). In brief, 30 honeysuckle plants were randomly selected and the soil around their roots (diameter = 40 cm, depth = 20 cm) was removed to identify larval species and calculate population abundance.

The first experiment was performed in a honeysuckle field in Fumin village (N35°15′23″, E117°40′54″) on August 26, 2020, at 1:30 pm, to determine which EPN species to apply against white grubs and at which rates. The sandy soil in the field had a water content of 8.09 ± 0.53%. The day was sunny, air temperature was 29°C and soil temperature was 27°C at a depth of 5 cm. The honeysuckle plants in the experiment field were 3 years old. Each plant covered an area of ∼1.15 m^2 (diameter = 1.21 m). Each experimental plot had an area of 48 m^2 (15 m × 3.2 m) with a 1.6-m buffer space set between plots and containing 36 honeysuckle plants spaced 1.6-m apart within a row. The white grub species present in the experiment field were *H. oblita, Brahmina faldermanni,* and *Serica orientalis* with a ratio of 5: 4: 6. The population density was 5.67 ± 0.61 larvae per plant, and the larvae were mainly in the first, second, and third instar with a ratio of 1: 7: 2. *S. longicaudum*, *S. glaseri*, and *H. bacteriophora* treatments were applied at 3.0×10^9, 1.5×10^9, and 7.5×10^8 IJs/ha, respectively.

The second experiment was conducted in Nanwan village (N35°16′56″, E117second) on August 18, 2021, at 4:30 pm, to assess the efficacy of EPNs against white grubs at the selected application rate and the protection provided to honeysuckle plants. The sandy soil in the fieldhad a water content of 6.22 ± 0.34%; the day was sunny with an air temperature of 27°C and soil temperature of 26°C at a depth of 5 cm. The honeysuckle plants were 3 years old. Each plant covered an area of ∼1.04 m^2 (diameter = 1.15 m). Each experimental plot had an area of ∼100 m^2 (33.3 m × 3.0 m) with a 1.5-m buffer space set between plots and contained 75 honeysuckle plants spaced 1.6 m apart within a row. The white grub species present in this experiment field were *H. oblita*, *S. orientalis*, and *Hoplosternus incanus* with a ratio of 8: 9: 2. The population density was 3.20 ± 0.05 larvae per plant and the larvae were mainly in the first, second, and third instar with a ratio of 1: 8: 1. *S. longicaudum*, *S. glaseri*, and *H. bacteriophora* treatments were applied at 1.5×10^9 IJs/ha.

In both experiments, phoxim (EC 48%, Shandong United Pesticide Industry Co. Ltd, Jinan, China) at a dosage of 4,500 mL/ha was used as a positive control. Water without IJs or insecticide was set as a negative control. In the first and second experiments, 15 L and 30 L of water, respectively, containing different concentrations of IJs or phoxim were sprayed on the soil around each plant root. A similar volume of only water was used for the control experiment. No additional irrigation or other insecticides were supplied. Each treatment was conducted in four replicates (plots) and all the plots were arranged in a randomized complete block design. Throughout both experiments, soil temperature at a depth of 5 cm ranged from 16 to 25°C.

White grub populations were monitored 7, 21, and 42 d after treatment (DAT) in both experiments. In the second experiment, plants selected for the larval abundance were excluded; the number of dead plants and total plants in each plot were determined on May 15, 2022, to calculate plant mortality.

EPN persistence in the soil was evaluated by assessing the mortality of yellow mealworm larvae buried in the soil samples 7, 14, 21, 28, 42 d after EPN application in both experiments. Soil sample (10 cm × 10 cm × 10 cm) around each plant roots was taken and five soil samples were taken from each plot. Then, 10 mealworm larvae were put in each soil sample and mortality was assessed 4 d later. Dead larvae were incubated in petri dishes with moist filter paper and were dissected 3 d later to estimate IJ invasion (Yan et al., 2013).

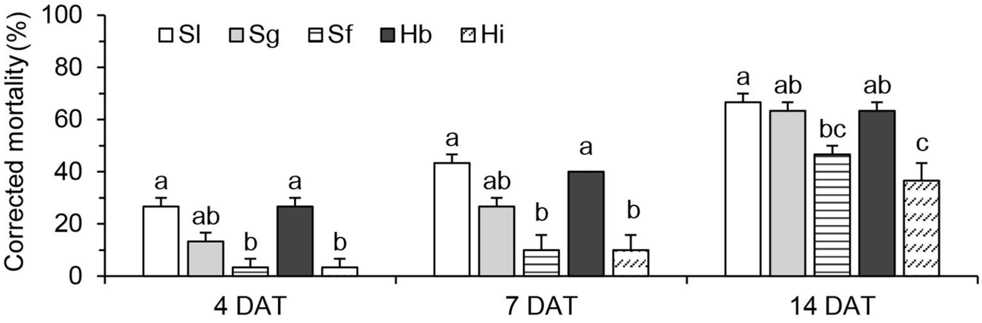

FIGURE 1
Corrected mortality of *Holotrichia oblita* Faldermann treated with *Steiernema longicaudum* X-7 (Sl), *S. glaseri* KG (Sg), *S. feltiae* SN (Sf), *Heterorhabditis bacteriophora* H06 (Hb), and *H. indica* LN2 (Hi) evaluated at 4, 7 and 14 DAT. Different letter(s) on the bars represent significant differences among treatments on the same day ($P < 0.05$, Tukey's test).

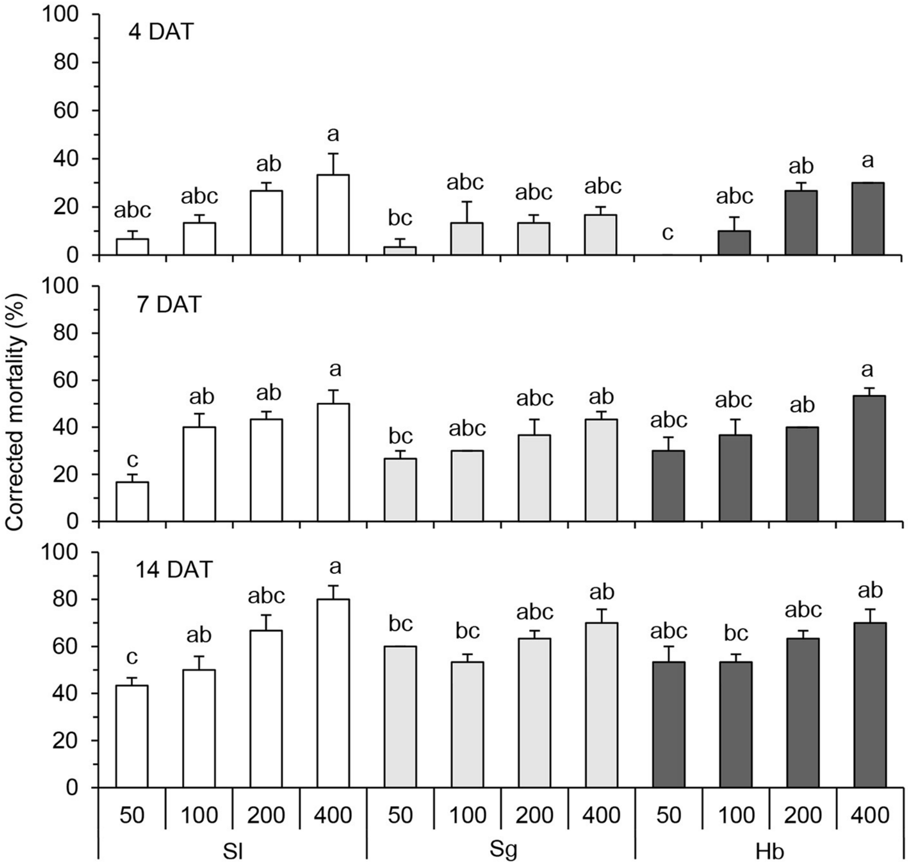

FIGURE 2
Corrected mortality of *Holotrichia oblita* Faldermann treated with *Steinernema longicaudum* X-7 (Sl), *S. glaseri* KG (Sg), and *Heterorhabditis bacteriophora* H06 (Hb) at rates of 50, 100, 200, and 400 IJs per larva evaluated at 4, 7, and 14 DAT. Different letter(s) on the bars represent significant differences among treatments on the same day ($P < 0.05$, Tukey's test).

2.4. Statistical analysis

The *H. oblita* and *T. molitor* bioassay data were corrected for control mortality using Abbott's formula (Abbott, 1925). The percentage reductions in white grubs in the field experiments were calculated based on Liu et al. (2007) and Guo et al. (2013, 2015). Plant mortality was calculated using the following equation:

$$P_{\mathrm{d}}(\%) = N_{\mathrm{d}}/N_{\mathrm{a}} \times 100,$$

where P_{d} is the percentage of dead plants in each plot, and N_{d} and N_{a} indicate the number of dead plants and the total number of plants in each plot, respectively.

TABLE 1 Percentage reduction in white grubs obtained from different treatments at 7, 21, and 42 days after treatment (DAT) in honeysuckle fields in Fumin (first experiment) and Nanwan (second experiment), Shandong, China.

Treatment[a]	% Grub reduction[b] at DAT		
	7	14	42
First experiment in Fumin			
Sl30	78.89 ± 4.29a	82.45 ± 3.41a	80.19 ± 4.04a
Sl15	76.48 ± 3.14a	78.35 ± 2.38ab	78.98 ± 2.64a
Sl7.5	65.86 ± 3.13ab	74.53 ± 4.65ab	74.57 ± 2.95ab
Hb30	76.28 ± 2.79a	80.24 ± 3.07a	81.21 ± 4.28a
Hb15	76.77 ± 2.75a	80.30 ± 3.26a	78.71 ± 3.88a
Hb7.5	66.64 ± 2.87ab	72.36 ± 3.09ab	77.32 ± 3.79a
Sg30	64.69 ± 3.28ab	67.47 ± 2.57ab	64.20 ± 3.79ab
Sg15	63.70 ± 3.99ab	67.65 ± 3.76ab	63.83 ± 3.73ab
Sg7.5	57.46 ± 4.13b	63.79 ± 2.36b	58.24 ± 3.15b
Phoxim	80.36 ± 3.87a	76.07 ± 3.13ab	74.68 ± 1.81ab
Second experiment in Nanwan			
Sl15	75.46 ± 3.36b	79.19 ± 4.54ab	74.91 ± 2.31a
Hb15	75.28 ± 2.78b	76.47 ± 5.36ab	72.34 ± 3.63a
Sg15	67.65 ± 1.77b	62.43 ± 2.85b	51.48 ± 5.94b
Phoxim	87.68 ± 2.56a	83.66 ± 3.68a	74.55 ± 4.75a

[a] Sl, *Steinernema longicaudum* X-7; Sg, *S. glaseri* KG; Hb, *Heterorhabditis bacteriophora* H06; 30 = 3.0×10^9 IJs/ha, 15 = 1.5×10^9 IJs/ha, 7.5 = 7.5×10^8 IJs/ha. Phoxim was applied at 4,500 mL/ha.

[b] Mean ± SE. Different letter(s) represent significant differences among treatments on the same DAT ($P < 0.05$, Tukey's test).

Arcsine square root transformation was applied to the percentage data before statistical analysis in SPSS 16.0 (SPSS Inc., Chicago, IL). Means were separated using Tukey's test and differences among means were considered significant at $P < 0.05$.

3. Results

3.1. Virulence of different EPN species against *H. oblita*

A significant difference was observed between white grub mortalities (hereafter referred to as "mortalities") caused by different EPN species (Figure 1). At 4 DAT, *S. longicaudum* and *H. bacteriophora* caused higher mortalities than *S. feltiae* and *H. indica*, while their values were not significantly different from the mortalities associated with *S. glaseri* ($F = 8.401$; $df = 4, 10$; $P = 0.003$). With time, an increase in mortality was observed in all treatments. Until 7 DAT, *S. longicaudum*, *S. glaseri*, and *H. bacteriophora*, caused significantly higher mortalities than *S. feltiae* and *H. indica* ($F = 6.110$; $df = 4, 10$; $P = 0.009$). At 14 DAT, the mortalities (63.33 ± 3.33% to 66.67 ± 3.33%) observed in the treatments with *S. longicaudum*, *S. glaseri*, and *H. bacteriophora*, were significantly higher than that by *H. indica* ($F = 9.357$; $df = 4, 10$; $P = 0.002$).

3.2. Effects of application rates on the virulence of superior EPNs

White grub mortalities varied with EPN application rates (Figure 2). Generally, the higher application rates were, the higher mortalities were obtained. White grub mortalities kept increasing with time. At 4 DAT, the mortalities caused by *S. longicaudum* at 400 IJs/larva and *H. bacteriophora* at 400 and 200 IJs/larva were significantly higher than that incurred by the same EPN species applied at lower rates ($F = 5.020$; $df = 11, 24$; $P < 0.001$). No significant difference was observed among the three application rates used for *S. glaseri*. At 14 DAT, the highest mortalities, ranging from 70.00 ± 5.77% to 80.00 ± 5.77%, were caused by *S. longicaudum*, *S. glaseri*, and *H. bacteriophora* at 400 IJs/larva ($F = 4.478$; $df = 11, 24$; $P = 0.001$).

3.3. Effects of EPN application in honeysuckle fields

The reduction in white grub population (hereafter referred to as "grub reduction") in the EPN- and phoxim-treated plots at different sampling times in the two experiments were shown in Table 1.

In the first experiment, the treatments with *S. longicaudum* and *H. bacteriophora* at all application rates showed high efficacy against white grubs. At 7 DAT, when compared insecticide phoxim, *S. longicaudum* and *H. bacteriophora* at all application rates caused similar grub reduction, while *S. glaseri* at 7.5 × 10^8 IJs/ha caused a significantly lower grub reduction ($F = 4.754$, $df = 9, 30$; $P = 0.001$). No significant difference was observed among the grub reduction from the treatments with the same EPNs at different application rates. However, the application of *S. longicaudum* and *H. bacteriophora* at the high rates of 3.0 × 10^9 and 1.5 × 10^9 IJs/ha and low rate of 7.5 × 10^8 IJs/ha caused > 76% and ≈ 65% grub reduction, respectively. However, grub reduction caused by both EPN species applied at 7.5 × 10^8 IJs/ha significantly increased over time, reaching 77.32 ± 3.79%. All rates of *S. longicaudum* and *H. bacteriophora* caused grub reductions ranging from 74.57 ± 4.65% to 81.21 ± 4.30% after 42 DAT, which were not significantly different from those obtained using phoxim, but significantly higher than the reductions obtained using *S. glaseri* at 7.5 × 10^8 IJs/ha ($F = 4.590$, $df = 9, 30$; $P = 0.001$).

In the second experiment, *S. longicaudum* and *H. bacteriophora* were approximately as efficient as phoxim in reducing white grub population. At 7 DAT, phoxim was more effective against white grubs when compared with the EPNs. The grub reductions caused by *S. longicaudum*, *S. glaseri*, and *H. bacteriophora* at 1.5 × 10^9 IJs/ha were 75.46 ± 3.36%, 67.65 ± 1.77%, and 75.28 ± 2.78%, respectively, significantly lower than that caused by phoxim ($F = 9.687$; $df = 3, 12$; $P = 0.002$). However, with time, the differencesbetween phoxim and the EPNs *S. longicaudum* and *H. bacteriophora*, were reduced to zero. Until 42 DAT, these two species and phoxim had the same efficacy, achieving grub reductions that were significantly higher than that caused by *S. glaseri* KG ($F = 6.292$; $df = 3, 12$; $P = 0.008$).

3.4. Effects of EPN application on plant mortality

Plant mortality in different treatment plots was shown in Figure 3. Plant death from plots treated with *S. longicaudum*, *S. glaseri*, *H. bacteriophora*, and phoxim were 0.84 ± 0.48%, 2.09 ± 0.41%, 0.83 ± 0.41%, and 0.85 ± 0.49%, respectively. No significant difference was observed among the treatments with the EPNs and phoxim. While the mortalities observed in plots treated with *S. longicaudum*, *H. bacteriophora*, and phoxim were all significantly lower than those in the control plots treated with water (4.19 ± 0.49%) ($F = 4.442$; $df = 4, 15$ $P = 0.014$).

3.5. EPN persistence

The mortalities of baited yellow mealworm larvae were calculated. *S. longicaudum*, *S. glaseri*, and *H. bacteriophora* were able to persist in the soil for 42 d after application (Table 2). Yellow mealworm mortalities ranged from 20.00 ± 4.08% to 45.00 ± 2.89% in the first field trail and from 27.50 ± 4.79% to 45.00 ± 2.89% in the second one. No significant difference was observed among treatments on the same sampling day (experiment 1: $F \leq 1.586$; $df = 8, 27$; $P \geq 0.176$; experiment 2: $F \leq 0.984$; $df = 2, 9$; $P \geq 0.411$).

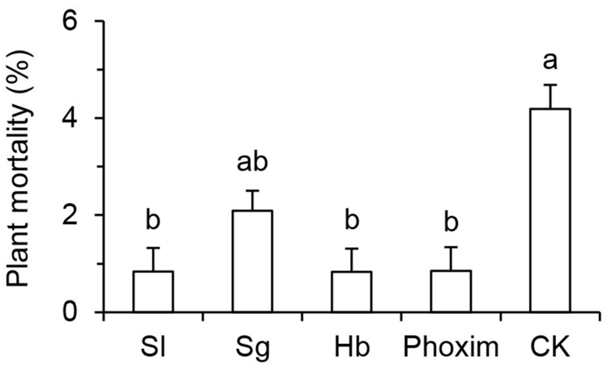

FIGURE 3
Plant mortality observed in different plots treated with *Steinernema longicaudum* X-7 (Sl), *S. glaseri* KG (Sg), and *Heterorhabditis bacteriophora* H06 (Hb) at 1.5 × 10^9 IJs/ha, and phoxim at 4,500 mL/ha evaluated on May 13, 2022. Different letter(s) on the bars represent significant differences among treatments and the water-based control (CK) ($P < 0.05$, Tukey's test).

4. Discussion

The screening of EPN species is critical to achieve a successful biocontrol of pests. Foremost, suitable EPN species must be matched with the target pest (Lacey and Georgis, 2012; Shapiro-Ilan and Dolinski, 2015). Among the EPN species tested via bioassay in this study, *S. longicaudum*, *S. glaseri*, and *H. bacteriophora* showed high virulence to *H. oblita*, which was congruent with previous studies that *S. longicaudum* (Li et al., 2007; Du et al., 2009; Guo et al., 2013), *S. glaseri*, and *H. bacteriophora* were highly pathogenic to a variety of scarab larvae (Grewal et al., 2005; Koppenhöfer and Fuzy, 2006). In contrast, our results showed that *S. feltiae* and *H. indica* were slightly virulent to grubs. The virulence of EPNs varies with different EPN species and target pests (Lacey et al., 2015) and little is known about that caused by *S. feltiae* and *H. indica* to scarab larvae. These two species have been reported to have a wide host range, with high virulence to fungus gnats (Zhao, 2013; Yan et al., 2019) and Lepidopterous pests (Lacey et al., 2015; Wang et al., 2021). Their lack of virulence to scarab larvae maybe partly due to their failure in overcoming host defenses (Wang et al., 1995; Lara-Reyes et al., 2021).

Although we firstly screened EPN species through laboratory bioassays to narrow down the candidates, the importance of confirming the virulence determined via bioassay by conducting subsequent filed trials cannot be overemphasized (Shapiro-Ilan et al., 2012b). In the honeysuckle fields treated in this study, the efficacy of *S. glaseri* against white grubs was not satisfactory. Although this was the first EPN species used to control white grubs at large scales (Gaugler et al., 1992), studies have shown that its field efficacy has deteriorated (Selvan et al., 1994; Converse and Grewal, 1998). Long-term laboratory culture may be one of the main factors responsible for its reduced performance (Converse and Grewal, 1998; Lee et al., 2002). Moreover, the potential virulence of *S. glaseri* against other white grub species co-occurring in same field may be another factor affecting its field efficacy. This virulence remains

TABLE 2 Corrected mortality of *Tenebrio molitor* bait larvae in soil samples collected from the first (Fumin) and second (Nanwan) experiments conducted in Shandong, China at 7, 14, 21, 28, and 42 DAT.

Treatment[a]	% Grub reduction[b] at DAT				
	7	14	21	28	42
First experiment in Fumin					
Sl30	32.50 ± 4.79	45.00 ± 2.89	37.50 ± 2.50	37.50 ± 9.47	37.50 ± 2.50
Sl15	25.00 ± 2.89	37.50 ± 2.50	25.00 ± 5.00	35.00 ± 5.00	25.00 ± 5.00
Sl7.5	37.50 ± 2.50	25.00 ± 5.00	35.00 ± 6.46	37.50 ± 2.50	35.00 ± 6.46
Hb30	25.00 ± 6.46	32.50 ± 4.79	32.50 ± 4.79	35.00 ± 6.46	42.50 ± 4.79
Hb15	35.00 ± 5.00	32.50 ± 4.79	35.00 ± 2.89	30.00 ± 5.77	30.00 ± 7.07
Hb7.5	22.50 ± 2.50	35.00 ± 5.00	40.00 ± 4.08	35.00 ± 2.89	27.50 ± 6.29
Sg30	30.00 ± 4.08	27.50 ± 4.79	35.00 ± 5.00	27.50 ± 4.79	32.50 ± 7.50
Sg15	30.00 ± 7.07	30.00 ± 5.77	37.50 ± 2.50	32.50 ± 4.79	35.00 ± 5.00
Sg7.5	25.00 ± 6.46	27.50 ± 4.79	27.50 ± 7.50	30.00 ± 4.08	20.00 ± 4.08
Second experiment in Nanwan					
Sl15	37.50 ± 4.79	42.50 ± 4.79	45.00 ± 2.89	30.00 ± 4.08	32.50 ± 4.79
Hb15	32.50 ± 2.50	37.50 ± 4.79	37.50 ± 4.79	27.50 ± 8.54	35.00 ± 5.00
Sg15	35.00 ± 2.89	37.50 ± 4.79	40.00 ± 4.08	32.50 ± 4.79	27.50 ± 4.79

[a] Sl, *Steinernema longicaudum* X-7; Sg, *S. glaseri* KG; Hb, *Heterorhabditis bacteriophora* H06; 30 = 3.0×10^9 IJs/ha, 15 = 1.5×10^9 IJs/ha, 7.5 = 7.5×10^8 IJs/ha.

[b] Mean ± SE. No significant difference was observed among the corrected mortalities obtained from different treatments on the same day (First experiment, $F \leq 1.586$; df = 8, 27; $P \geq 0.176$; Second experiment, $F \leq 0.984$; df = 2, 9; $P \geq 0.411$, Tukey's test).

to be determined, as in this study we only assessed the virulence against the larvae of *H. oblita.*

Unlike *S. glaseri* KG, both *S. longicaudum* and *H. bacteriophora* achieved an acceptable level of grub control in the treated honeysuckle fields, where multiple species of white grubs co-occurred. To ensure a successful control, it is important that EPN species are highly pathogenic to several grub species, as these have overlapping geographic ranges and may often co-occur in the same fields (Grewal et al., 2004). *Steinernema longicaudum* and *H. bacteriophora* have been shown to perform well against different white grub species; for example, *S. longicaudum* proved to be effective against *Polyphylla gracilicornis* (Fan, 2015) and *Holotrichia ovata* (Zhang et al., 2006) in bioassays, and against *Exomala orientalis* in turf grass (Lee et al., 2002) and *H. parallela* in peanut fields (Guo et al., 2013), while *H. bacteriophora* performed well against *Popillia japonica* in turf grass (Koppenhöfer and Fuzy, 2003a,b; Grewal et al., 2004; Torrini et al., 2020), *H. parallela* (Guo et al., 2013), and *H. oblita* (Guo et al., 2015) in peanut fields. Although we did not test the virulence of either EPN to the white grub species mentioned above via bioassay, we believe that both are suitable to control them based on the results of the present study and the good field performance reported in previous studies.

In addition to the suitability of EPN species, adequate environmental conditions, especially in terms of soil moisture, are considered as another important factor in EPN application (Kaya, 1990; Shapiro-Ilan et al., 2006a). The honeysuckle trees in this study were planted in hill fields with sandy soil characterized by poor water retention. However, according to our data, the soil moisture detected during the experimental period ranged from 8.07 to 16.33%, and EPNs could establish well in this soil. The honeysuckle trees in the experiment fields were 3-years-old, with lush vines covering the ground. We speculated that the good level of shade and frequent rainfall in summer and autumn contributed to the adequate soil moisture. Generally, white grub outbreaks in honeysuckle are persistent (Li, 2022). In this study, EPNs could reduce white grub populations in the long term in the fields, which indicated that soil conditions, including soil moisture, texture, and temperature (16–25°C), favored EPN establishment, dispersal, and contact with hosts (Guo et al., 2015).

In the present study, plant mortalities in the plots treated with *S. longicaudum*, *H. bacteriophora*, and phoxim were significantly reduced by a rate of ≈80% compared with the values observed in the control plots treated with water. This indicated that the reduction in white grubs obtained through the above-mentioned treatments could lower plant mortality. To improve the curative qualities of honeysuckle flowers, more attention should also be paid to ecological planting. In particular, the application of EPNs to control pests dwelling below the ground is of great significance for ecological planting, not only for biological control purposes, but also to enhance plant defenses (Helms et al., 2019). Further studies should focus on the effects of EPNs on the soil system and the quality of honeysuckle flowers after EPN application.

In our study, both *S. longicaudum* and *H. bacteriophora* treatments performed well against white grubs in honeysuckle fields. However, *H. bacteriophora* may be considered as a more promising agent due to its relatively lower production cost (Guo et al., 2013) and higher stability under unfavorable conditions (Yan et al., 2010) compared with *S. longicaudum*. The EPN application rate is of paramount importance, varying across target pests and environmental settings (Shapiro-Ilan and Dolinski, 2015).

Generally, higher application rates could enhance the efficacy of the treatments to some degree, achieving results within a shorter period of time (Guo et al., 2015). This would also entail an increase in costs; however, applying lower EPN rates will increase the risk of low efficacy against white grubs (Shapiro-Ilan et al., 2006a), as suggested by the data in our first experiment. Our results showed that 1.5 × 10^9 IJs/ha would be an optimal application rate for honeysuckle fields, considering that higher rates did not determine a greater reduction in white grubs at all.

In summary, the present study highlighted the potential of using EPNs against white grubs in honeysuckle fields. Additional studies are needed on how to accelerate the effects of EPN treatments through the joint application of EPNs and other entomopathogenic agents, such as *Metarhizium anisopliae* and *Beauveria bassiana*, among others, which will improve efficacy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

XL, WG, and YY conceived and designed the research. XL, SL, LL, HC, and YS conducted experiments. XL analyzed the data and produced a draft of the manuscript. XM, WG, YY, JW, XF, and ZS provided comments on various drafts. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Fang Ouyang,
Chinese Academy of Sciences (CAS), China

REVIEWED BY
Huipeng Pan,
South China Agricultural University, China
Wendy-Ann P. Isaac,
The University of the West Indies St. Augustine, Trinidad and Tobago

*CORRESPONDENCE
Yaobin Lu
✉ luybcn@163.com
Xiaowei Li
✉ lixiaowei1005@163.com

†These authors have contributed equally to this work

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Development of attractants and repellents for *Tuta absoluta* based on plant volatiles from tomato and eggplant

Tingting Chen[1,2†], Limin Chen[1,3†], Jinchao Wang[1], Jianghui Cheng[1], Songwang Yi[1], Muhammad Hafeez[1], Shuxing Zhou[1], Yuanxi Li[2], Xiaowei Li[1]* and Yaobin Lu[1]*

[1]State Key Laboratory for Managing Biotic and Chemical Threats to Quality and Safety of Agro-products, Key Laboratory of Biotechnology in Plant Protection of MOA of China and Zhejiang Province, Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China, [2]College of Plant Protection, Nanjing Agricultural University, Nanjing, China, [3]Lishui Academy Institute of Agricultural and Forestry Sciences, Lishui, Zhejiang, China

Introduction: *Tuta absoluta* is currently considered one of the most devastating invasive pests of solanaceous plants worldwide, causing severe damage to the tomato industry. Insects use volatile organic compounds (VOCs) to locate host plant for feeding and oviposition. Those VOCs could be developed as lures for pest monitoring and control.

Methods: In this study, the differentially accumulated VOCs between the preferred host (tomato) and non-preferred host (eggplant) were analyzed by GC–MS method, and their roles on female *T. absoluta* host selection and egg laying behaviors were investigated using electroantennography (EAG), olfactometer and cage experiments.

Results: A total of 39 differentially accumulated VOCs were identified in tomato and eggplant. Strong EAG signals were obtained in 9 VOCs, including 5 VOCs highly accumulated in tomato and 4 VOCs highly accumulated in eggplant. Further olfactometer bioassays showed that 4 compounds (1-nonanol, ethyl heptanoate, ethyl octanoate and o-nitrophenol) were attractive to *T. absoluta* females, while 5 compounds (2-phenylethanol, 2-pentylfuran, trans,trans-2,4-nonadienal, 2-ethyl-5-methylpyrazine and trans-2-nonenal) were repellent, indicating that VOCs from host plants play important roles in host plant preferences. The attractive activities of 1-nonanol and ethyl octanoate, as well as the repellent activities of trans,trans-2,4-nonadienal and trans-2-nonenal, were further confirmed in cage experiments.

Discussion: In this study, two attractants and two repellents for *T. absoluta* were developed from plant released VOCs. Our results could be useful to enhance the development of eco-friendly and sustainable pest management strategies for *T. absoluta*.

KEYWORDS

***Tuta absoluta*, tomato, eggplant, VOCs, oviposition preferences, attractants, repellents**

1. Introduction

The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera, Gelechiidae), an invasive pest native to Peru, South America, has become one of the most devastating pests of solanaceous plants worldwide (Biondi et al., 2018). It can infest host plants at all developmental stages, with the larvae mining and feeding on leaves, stems and fruits, causing crop losses up to 80–100% in the area without timely control measures (Desneux et al., 2010). After invading Europe in 2006, this pest spread quickly to Afro-Eurasian supercontinent, and now has been recorded in more than 90 countries and regions outside South America (Biondi et al., 2018; Desneux et al., 2022). In Asia, *T. absoluta* has been found in many countries (Campos et al., 2017; Han et al., 2019), including countries on the northwestern and southwestern border of China, e.g., Tajikistan, Kyrgyzstan, India, Nepal, etc. (Campos et al., 2017; Sankarganesh et al., 2017; Uulu et al., 2017; Saidov et al., 2018). Recently, this pest was found in northwest China in Xinjiang (Li D. et al., 2019; Li X. W. et al., 2019; Zhang et al., 2019) and southwest China in Yunnan (Zhang et al., 2020), and has quickly spread to 14 provinces in China mainland, poses a significant threat to China's tomato production (Zhang et al., 2021).

Currently, chemical control is still the main control method for *T. absoluta* implemented in its native ranges and invaded countries to counter its great threat to agricultural production (Guedes et al., 2019). It has been reported that the application of insecticides could prevent the occurrence and spread of *T. absoluta* to some extent (Silvério et al., 2009). However, the control effects of insecticides is limited because of the larval endophytic feeding behavior which makes *T. absoluta* a difficult target for insecticide sprays (Guedes and Siqueira, 2012). In addition, due to the intensive use of insecticides, *T. absoluta* has become resistant to many chemical classes of insecticides, including organophosphates (Siqueira et al., 2000b; Lietti et al., 2005; Haddi et al., 2017; Barati et al., 2018), pyrethroids (Haddi et al., 2012; Biondi et al., 2015), spinosyn (Campos et al., 2014, 2015), avermectins (Siqueira et al., 2001; Silva et al., 2016), cartap (Siqueira et al., 2000a), benzoylureas (Silva et al., 2011), indoxacarb (Roditakis et al., 2018) and diamides (Silva et al., 2019). Alternative control strategies should therefore be used within the context of integrated pest management (IPM) for this destructive pest.

In response to insect herbivory, plants release volatile compounds that may serve as protective substances as well as mediators of interactions with other plants, microbes, and animals. Plant-released semiochemicals are promising eco-friendly pest management methods that has been widely used as a sustainable alternative for synthetic insecticides (Dudareva et al., 2006; Shrivastava et al., 2010; Beck et al., 2017). In the process of co-evolution between insects and plants, there is a corresponding interaction between insects and plants. The most primitive ecological relationship is that insects select their compatible host plants, while the phytoconstituents of host plants are one of the direct causes of host plant-insect interaction (Thompson, 1988). Plants could release different classes of volatile organic compounds (VOCs) into the external environment during their growth and development, which enables plants to generate defense signals and communicate with each other (Baldwin et al., 2006; Heil and Silva Bueno, 2007). Plant VOCs also play important roles in plant-insect interactions, affecting insect feeding, mating and egg-laying (Bruce et al., 2005). Insects use plant volatiles to locate plant hosts for feeding and oviposition (Kuhnle and Muller, 2011; Wynde and Port, 2012). Those VOCs could be developed as lures for pest monitoring and control (Shrivastava et al., 2010). On the other hand, many plants have developed counter strategies to defend themselves against these insects, including repellent VOCs, which could be developed into repellents to reduce pest populations on target crops (War et al., 2012).

The use of plant chemicals (VOCs and non-volatile secondary metabolites) for pest control has been reported for *T. absoluta*. For instance, it has been reported that the extracts of jojoba, *Simmondsia chinensis*, can effectively control *T. absoluta* (Abdel-Baky and Al-Soqeer, 2017). Essential oils of three *Satureja* species, *S. khuzestanica*, *S. bachtiarica*, and *S. rechingeri*, had fumigant toxicity on *T. absoluta*, with geraniol the main component of all essential oils (Rahmani and Azimi, 2021). Shared volatile compounds from different hosts [a blend consisting of limonene (16.64%), β-ocimene (1.84%), α-terpinene (12.17%),δ-eIemene (4.29%) and (E)-β-caryophyllene (6.78%)] could attract female *T. absoluta* (Msisi et al., 2021). Consequently, understanding the plant chemicals involved in *T. absoluta*-host plant interactions could be useful for the development of a new strategy for the control of this pest.

Tuta absoluta is oligophagous and can survive and reproduce normally on potatoes, tobacco and other Solanaceae crops (Arnó et al., 2019). Nevertheless, it has been found that *T. absoluta* had a strong preference for tomatoes among host plants, and volatile chemical signals played important roles in its host plant preferences (Subramani et al., 2021). Similarly, in our previous study, it has been found that the number of eggs laid by *T. absoluta* was significantly higher on tomatoes than on eggplants (Chen et al., 2021). In this study, we further analyzed the differentially accumulated VOCs between these two host plants, and their roles on female host selection and egg laying behaviors were investigated by using electroantennography (EAG), olfactometer and cage experiments. The VOCs with attractive and repellent activities could be used to develop new control strategies for this pest.

2. Materials and methods

2.1. Plant materials and insects

Seeds of tomato (variety Zhefen 202) and eggplant (variety Zheqie NO.1) were sown in the coconut coir for germination. After the two-leaf stage, seedlings were individually transplanted into plastic pots (7 cm long, 7 cm wide and 9 cm high) and placed in an insect-free greenhouse (26 ± 5°C, 60% ± 5% RH, 16 L:8 D photoperiod). The plants were watered at regular intervals and 1 g of water-soluble fertilizer containing 18 macro-elements (OMEX, 18–18-18) was applied to each plant. Plants at the 5-leaf stage were used for host plant VOC collection and egg-laying experiments.

Tuta absoluta populations were collected in 2018 from tomato fields in Yili, Xinjiang, and were continuously reared in an artificial climate chamber (25 ± 1°C, 60% ± 5% RH, 16 L:8 D photoperiod) on tomato plants.

2.2. Headspace solid-phase microextraction coupled to gas chromatography–mass spectrometry (HS-SPME/GC–MS)

When the healthy host plants (tomato and eggplant) were at the 5-leaf stage, the third leaf from the top was selected, the veins were removed, approximately 1 g of each sample was lyophilized in liquid nitrogen (LN) and subsequently stored in a − 80°C freezer. Samples were later pulverized in liquid nitrogen and vortexed to mix evenly. The samples were moved into a headspace bottle with fully automatic headspace solid-phase microextraction (HS-SPME) (Lee et al., 2007). The gas chromatography-mass spectrometer (GC–MS) was used to identify terpenoids, benzene ring types and phenylpropanoids, fatty acid derivatives and other volatiles. The volatile content was determined by the headspace collection method or extraction method. The SPME readings were taken at 250°C aging temperature; 5 min aging time; 60°C heating temperature; 10 min heating time; 20 min adsorption time; 5 min desorption time; and 5 min aging time after sample injection. The original data file obtained by GC–MS analysis was first extracted using the MassHunter software (Agilent) (Yuan et al., 2022). Three samples were collected and tested both for tomato and eggplant.

2.3. Electroantennographic (EAG) responses of *T. absoluta* females to VOCs

To test whether differentially accumulated VOCs between tomato and eggplant contribute to host plant preference of *T. absoluta*, EAG responses of *T. absoluta* females and males to 20 differentially accumulated VOCs were determined using the EAG detection system (Stimulus Air Controller CS-55 and SYNTECH IDIC2; Syntech, Hilversum, the Netherlands). The 20 VOCs, which were selected according to the principal component analysis and the characteristics of VOCs, were as follows: 1-nonanol, 2-phenylethanol, 2-isopropyl-3-methoxypyrazine, ethyl heptanoate, ethyl octanoate, 1,4-diethylbenzene, o-nitrophenol, which were highly accumulated in tomato; benzyl alcohol, 2-pentylfuran, benzaldehyde, trans,cis-2,6-nonadienal, trans,trans-2,4-nonadienal, furfural, trans-2-hexen-1-al, trans,trans-2,4-heptandienal, isophorone, 2-s-butylphenol, 4-hexen-3-one, 2-ethyl-5-methylpyrazine, and trans-2-nonenal, which were highly accumulated in eggplant. The synthetic standards of the above VOCs were purchased from Merck and Shanghai Aladdin Biochemical Technology Co.

The standard compounds were diluted in a gradient with paraffin oil to four concentrations (0.1, 1, 10, and 100 mg/mL), 10 μL of each was applied to a piece of filter paper (5 mm × 2 cm), which was placed into Pasteur pipette 10 min before testing. 10 μL of paraffin oil was used as a control stimulus. The stimulus was made by introducing the test volatile to the antenna at a flow rate of 25 mL/min for 2 s and with an interval of 1 min for the next stimulus. For each test chemical, paraffin oil was used as control. The test order was paraffin oil, the test compound, and paraffin oil. The test compound of each concentration was performed on five females and males. Relative EAG values of *T. absoluta* were reported as the percentage of the response to paraffin oil.

2.4. Olfactometer bioassay

The responses of *T. absoluta* females and males to 9 volatile compounds with strong EAG responses, including 5 highly accumulated in tomato (1-nonanol, 2-phenylethanol, ethyl heptanoate, ethyl octanoate and o-nitrophenol) and 4 highly accumulated in eggplant (2-pentylfuran, trans,trans-2,4-nonadienal, 2-ethyl-5-methylpyrazine and trans-2-nonenal), were tested by using Y-tube olfactometer. The glass Y-tube is with a 3-arm structure, which consists of a 60-mm-long base tube and two 60-mm-long arms. The two arms were separated from each other at an angle of 90°. Teflon tubes were used to connect the components of the olfactometer apparatus. Air was pumped into the apparatus by an electromagnetic air pump, filtered by activated carbon, and split into two air streams at a flow rate of 60 ml/s. Before each test, the apparatus was rinsed with pure ethanol and dried in an oven (120°C).

Tuta absoluta females and males of mixed ages (2–4 days) were used for Y-tube olfactometer bioassays. The bioassays were conducted in a dark room at 25 ± 1°C and 60% ± 5% RH. The light was provided by an LED lamp located in the ceiling directly above the Y-tube. Solutions of each VOC compounds were prepared in paraffin oil at a gradient of four concentrations (0.1, 1, 10, and 100 mg/mL), and 10 μL was pipetted onto a piece of clean filter paper (1 × 1 cm), which was then transferred to a glass flask as the test odor source. Filter paper with 10 μl of paraffin oil in a glass flask was used as a control odor source.

Tuta absoluta females and males were individually transferred to the base tube of the Y-tube and their choice was recorded within 5 min. When the tested individual crossed half-length of either arm, the “effective choice” was recorded. If the tested individual did not cross half-length of either arm within 5 min, the “no choice” was recorded. To prevent the effects of light, the Y-tube arms were swapped after every 5 insects. The experiment was repeated five times, with 20 individuals each time.

2.5. Cage experiments

Cage experiments were conducted in a climate chamber (25 ± 1°C, 60% ± 5% RH, 16 L: 8 D photoperiod) to test the responses of *T. absoluta* females to 4 VOCs (1-nonanol, ethyl octanoate, trans,trans-2,4-nonadienal and trans-2-nonenal) that showed the highest attractive or repellent activities to *T. absoluta* females in olfactometer bioassays. In each cage, six five-leaf stage tomato plants were placed equally along two opposite sides of the cage, with three plants along each side. The concentrations of standard compounds that showed the highest attractive or repellent activities to *T. absoluta* were used in cage experiments. The standard compounds of selected VOCs were dissolved in hexane at the required concentration. A Rubber septum with 10 μl diluted standard compounds of selected VOCs was hung on each of the three plants on one side of the cage. Rubber septa with 10 μl of hexane were used as control, and were hung on plants on the other side of the cage. Thirty 2 to 4 days old females of *T. absoluta* were released from the middle of the cage. After 48 h, the number of *T. absoluta* eggs on all leaves of each tomato plant was counted. The experiment was repeated twice for each tested VOC compound.

2.6. Statistical analysis

Quality control (QC) analysis was conducted before data were obtained from GC–MS to confirm the reliability of the data before the overall analyses. The QC sample was prepared by mixing sample extracts for insertion into every three samples to monitor the changes in repeated analyses. Data matrices with the intensity of the metabolite features from the samples were uploaded to the Analyst software (version 1.6.1; AB Sciex, Canada) for statistical analyses. The partial least squares discriminant analysis (PLS-DA) was performed to maximize the metabolome differences between sample pairs. The relative importance of each metabolite to the PLS-DA model was tested using the variable importance in projection (VIP) as a parameter. Metabolites with $VIP \geq 1$ and fold change ≥ 2 or fold change ≤ 0.5 were considered differential metabolites for group discrimination (Chong et al., 2018). PCA and Ward's hierarchical clustering heatmap were performed using R software (version 3.3.2).[1] Consequently, a metabolic pathway was constructed according to KEGG[2]; and pathway analysis was performed using MetaboAnalyst[3] based on the change in metabolite concentration compared with the corresponding controls.

EAG data were analyzed using one-way ANOVA followed by Turkey's Highest Significant Difference. Olfactometer data was analyzed using the Chi square test. The null hypothesis was that *T. absoluta* had 50: 50 distributions across the two arms of the olfactometer. Differences in the number of eggs on tomato plants with VOC compounds and solvent control were analyzed using Student *t*-test. Data analyses were performed by using SPSS (SPSS Inc., 2007, Chicago, IL) with $p \leq 0.05$.

3. Results

3.1. Analysis of differentially accumulated VOCs in tomato and eggplant

VOCs released from the two host plants (tomato and eggplant) collected by HS-SPME were identified by GC–MS. In total, one hundred and forty VOCs predominantly from alkanes (24), heterocyclic compounds (20), alcohol (16), aldehyde (16), terpenes (14), ketone (13), ester (11), aromatics (10), phenol (8), olefin (3), acid (2), ether (1), amine (1) and other (1) classes were detected in this study (Figure 1A; Supplementary Table S1). Thirty-nine differentially accumulated VOCs were identified between these two different host plants (Supplementary Table S2), of which, 15 VOCs were accumulated higher in tomato than in eggplant, which belong to alcohol (3), heterocyclic compound (3), terpenes (2), aromatics (2), ester (2), alkanes (1), aldehyde (1) and phenol (1). These VOCs might contribute to the higher attraction of tomato plants to *T. absoluta* females for host selection and oviposition. While 24 VOCs were accumulated higher in eggplant than in tomato, which belong to aldehyde (9), heterocyclic compound (5), alcohol (3), ketone (3), phenol (2), terpenes (2). These VOCs might have repellent activities to *T. absoluta*.

The 39 differentially accumulated VOCs were further evaluated using principal component analysis (PCA) to clarify that the differentially accumulated VOCs detected could be used to distinguish between the two host plants. PCA analysis (Figure 1B) indicated that the detected VOCs were divided into two groups, with significant differences between tomato and eggplant, suggesting significant differences in VOCs between the two host plants. Although tomato and eggplant could not be distinguished in the PC2 (vertical axis) principal component, a significant distinction between tomato and eggplant could be found in the PC1 (horizontal axis) principal component. The PC1 (horizontal axis) principal component explained 62.51% of the total variance between samples, while PC2 (vertical axis) explained only 16.88%. The variability between sample groups and the similarity within sample groups confirmed the differential accumulation of VOCs in the two host plants.

We performed a hierarchical clustering analysis of the differentially accumulated VOCs detected in tomato and eggplant samples (Figure 1C), which showed a high degree of similarity between the biological replicates within each host plant and significant differences between the tomato and eggplant samples. These results indicate the high quality of data from both sets of samples and the presence of significant differences in VOCs in the two host plants.

3.2. EAG responses of *T. absoluta* females to VOCs

To demonstrate that the differential accumulation of VOCs on the two host plants identified in this study does have effects on the egg-laying behavior of *T. absoluta* females, the EAG response of *T. absoluta* females and males to 20 differentially accumulated VOCs were initially screened. These 20 VOCs included 7 VOCs that were higher accumulated in tomatoes than in eggplants, namely 1-nonanol, 2-phenylethanol, 2-isopropyl-3-methoxypyrazine, ethyl heptanoate, ethyl octanoate, 1,4-diethylbenzene, o-nitrophenol, and 13 VOCs that were higher accumulated in eggplants namely benzyl alcohol, 2-pentylfuran, benzaldehyde, trans,cis-2,6-nonadienal, trans,trans-2,4-nonadienal, furfural, trans-2-hexen-1-al, trans,trans-2,4-heptandienal, isophorone, 2-S-butylphenol, 4-hexen-3-one, 2-ethyl-5-methylpyrazine, trans-2-nonenal.

The results showed that all the 20 VOCs triggered certain EAG responses of *T. absoluta* females (Figure 2), confirming that the selected VOCs might have some effects on host plant selection and egg-laying behavior of *T. absoluta* females. It is also noteworthy that among the 20 compounds tested, 9 VOCs, including 5 highly accumulated in tomato (1-nonanol, 2-phenylethanol, ethyl heptanoate, ethyl octanoate and o-nitrophenol) and 4 highly accumulated in eggplant (2-pentylfuran, trans,trans-2,4-nonadienal, 2-ethyl-5-methylpyrazine and trans-2-nonenal), caused significantly higher EAG responses in *T. absoluta* females. The EAG responses of *T. absoluta* males to 20 VOCs (Supplementary Figure S1) were roughly the same as females.

3.3. Olfactory responses of *T. absoluta* females to VOCs

Among the 9 VOCs in the olfactometer bioassays, 4 compounds (all higher in tomatoes than in eggplants) were attractive to *T. absoluta*

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[1] www.r-project.org

[2] https://www.genome.jp/kegg/

[3] https://www.metaboanalyst.ca/

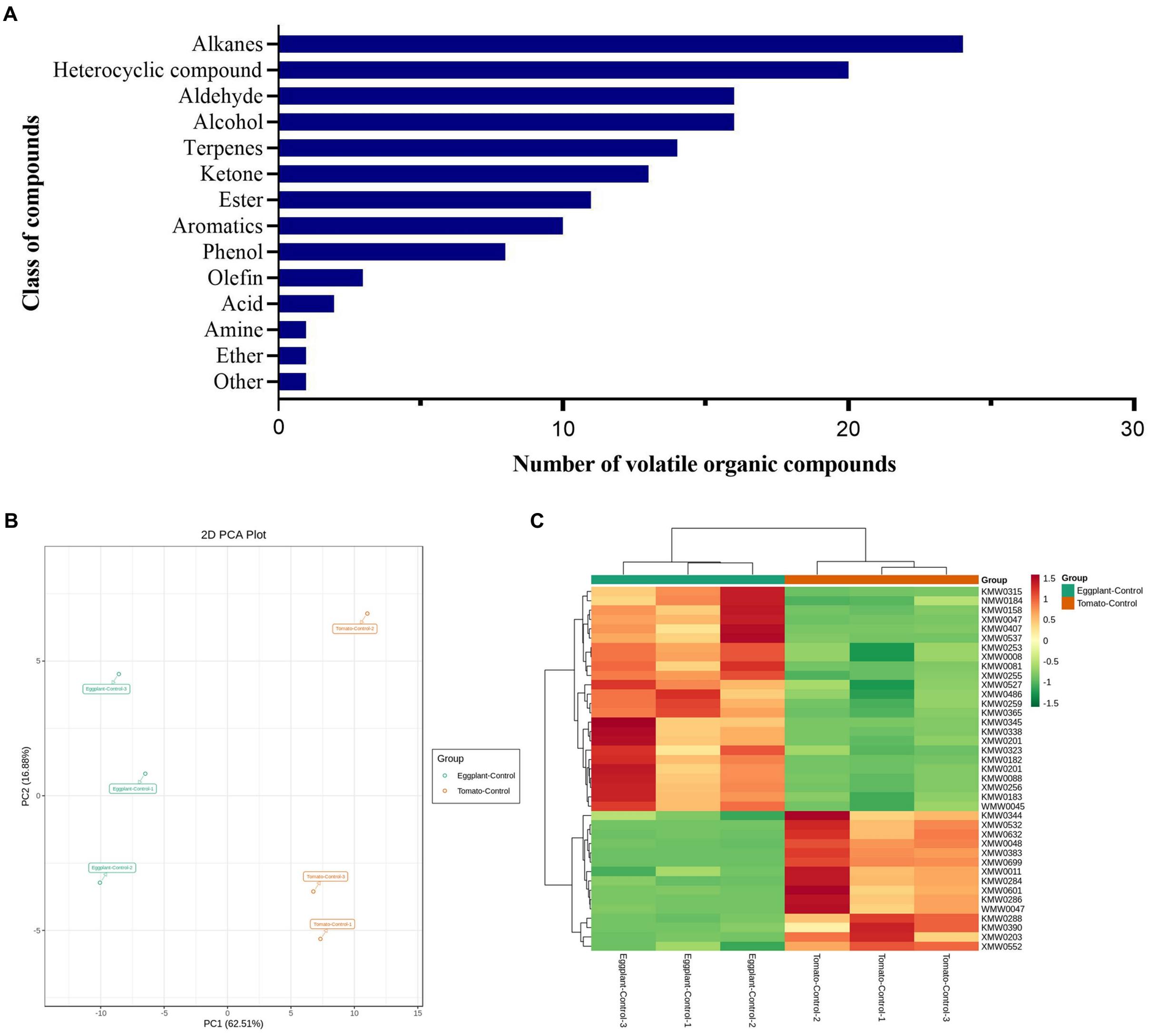

FIGURE 1
(A) Classes of volatile organic compounds identified of tomato and eggplant. **(B)** Principal component analysis (PCA) of differentially accumulated VOCs identified from tomato and eggplant leaves by headspace solid-phase microextraction (HS-SPME). **(C)** Heatmap clustering of 39 differentially accumulated VOCs identified from tomato and eggplant leaves.

females and 5 compounds (four of them were higher in eggplant than in tomato) were repellent (Figure 3). Specifically, 1-nonanol was shown to be attractive to *T. absoluta* females at doses of 1 and 10 μg, but had no significant attractive effect at doses of 100 and 1,000 μg. Ethyl heptanoate at a dose of 10 μg showed an attractive effect on *T. absoluta* females, but no significant effects at 1, 100 and 1,000 μg. Ethyl octanoate was attractive to *T. absoluta* females at 1, 10 and 100 μg, but there was no significant effect at 1000 μg. O-nitrophenol at a dose of 1 μg produced an attractive effect on *T. absoluta* females, but there were no significant effects at doses of 10, 100 and 1,000 μg. By contrast, 2-phenylethanol at 100 and 1,000 μg produced repellent effects on *T. absoluta* females, but no significant repellent activities were found at 1 and 10 μg doses. 2-pentylfuran at 1000 μg produced a repellent effect on *T. absoluta* females, but there was no significant repellent effect at 1, 10 and 100 μg. The trans,trans-2,4-nonadienal produced repellent effects on *T. absoluta* females at doses of 10, 100 and 1,000 μg, but no significant effect at 1 μg. 2-ethyl-5-methylpyrazine produced repellent effects on *T. absoluta* females at doses of 100 and 1,000 μg, but not repellent at 1 and 10 μg. The trans-2-nonenal produced repellent effects on *T. absoluta* females at four doses of 1, 10, 100 and 1,000 μg. Notably, 1-nonanol and ethyl octanoate, which were more abundant in tomatoes compared to eggplants, showed significant attractive activities to *T. absoluta* females, while trans,trans-2,4-nonadienal and trans-2-nonenal, which were more abundant in eggplants than tomatoes, showed significant repellent activities to *T. absoluta* females. The behavioral responses of *T. absoluta* males to these nine VOCs were highly consistent with females (Supplementary Figure S2).

3.4. Effects of VOCs on the oviposition behavior of *T. absoluta*

Results from cage experiments showed that 1-nonanol, ethyl octanoate, trans,trans-2,4-nonadienal and trans-2-nonenal could significantly influence the number of eggs laid by *T. absoluta* on the

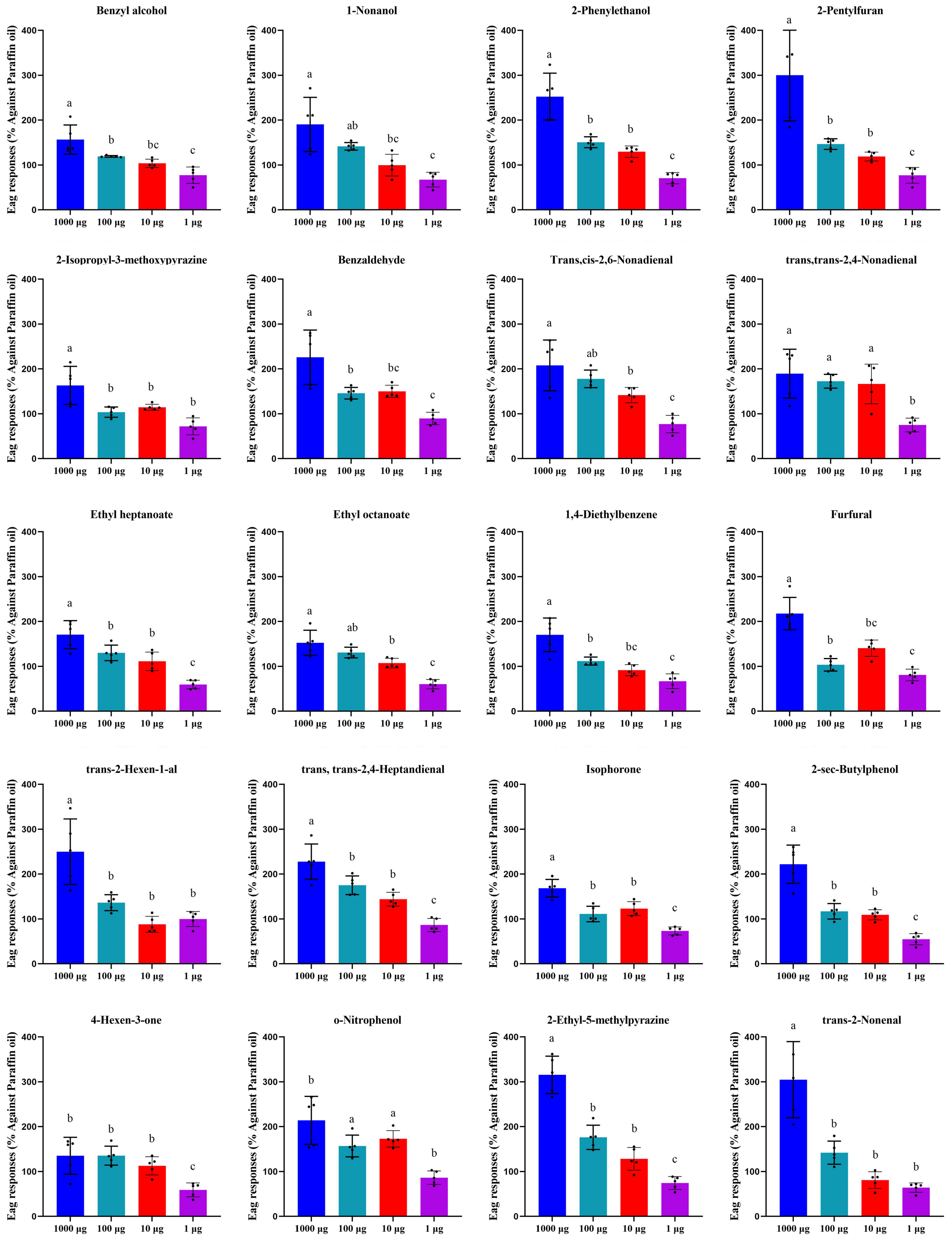

FIGURE 2
Electroantennographic (EAG) responses of *T. absoluta* females to 20 volatile compounds. The bar represents the standard error, and the different letters above each bar indicate Turkey's highest significant difference at $p<0.05$.

host plants (Figure 4). Specifically, *T. absoluta* females laid significantly more eggs on tomato plants with 1-nonanol by 91.6% compared to control plants with hexane. Similarly, *T. absoluta* females produced significantly more eggs on tomato plants with ethyl octanoate by 245.2% compared to control plants with hexane. By contrast, *T. absoluta* females produced 71.9% fewer eggs on tomato plants with

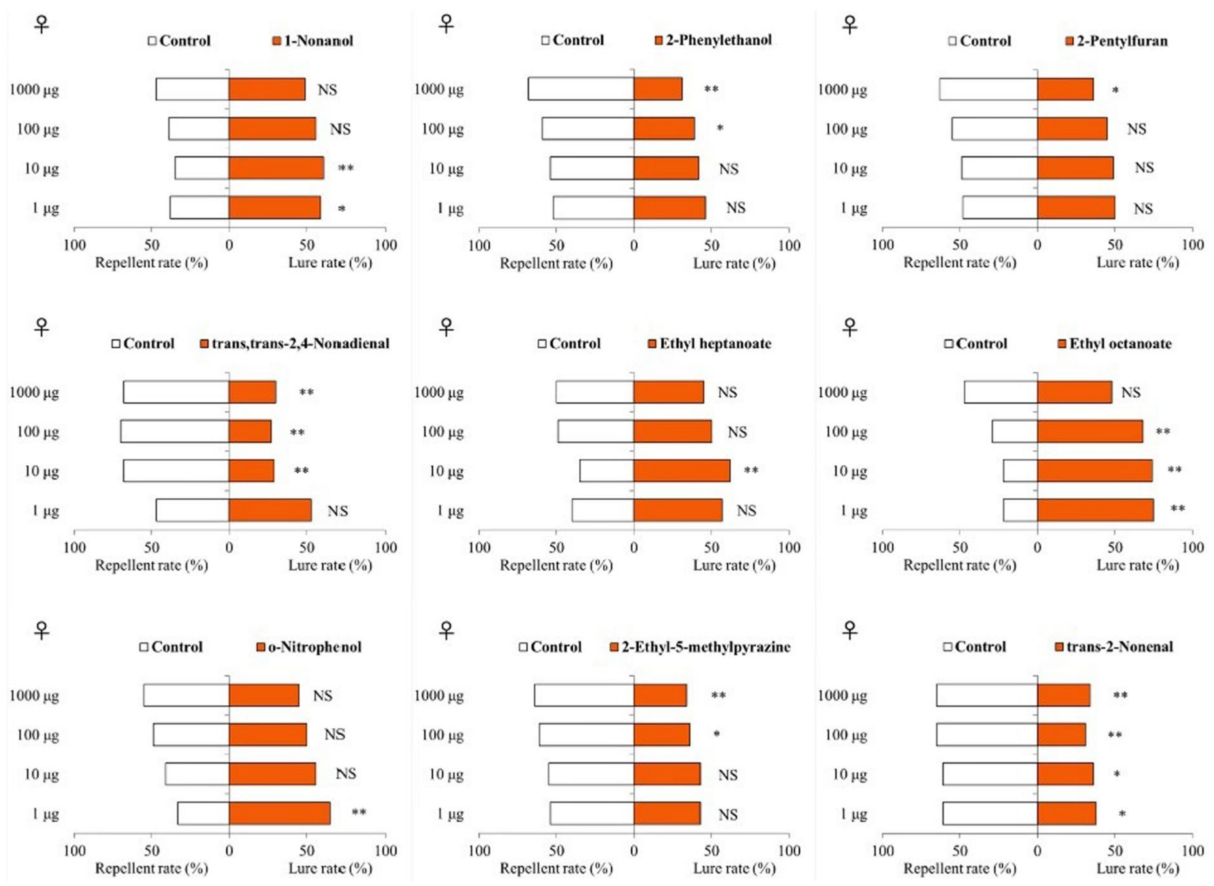

FIGURE 3
Responses of female *T. absoluta* to nine VOCs in a Y-tube olfactometer. NS indicates no significant difference; asterisks indicate significant differences (*p<0.05; **p<0.01).

trans,trans-2,4-nonadienal compared to control plants with hexane. *T. absoluta* females produced 35.8% fewer eggs on tomato plants with trans-2-nonenal compared to control with hexane. These results suggest that 1-nonanol and ethyl octanoate had significant attractive effects on the oviposition choice of *T. absoluta* females. On the contrary, trans,trans-2,4-nonadienal and trans-2-nonenal had repellent effects on the oviposition choice of *T. absoluta* females.

4. Discussion

Compared with polyphagous insects, oligophagous insects usually have a much stronger preference for selecting suitable host plants (Gripenberg et al., 2010). The dispersal ability of *T. absoluta* larvae is limited, consequently, host plant selection of *T. absoluta* females often determines the food source of their offspring at the larval stage (Silva et al., 2021). Results from our previous study showed that *T. absoluta* females showed significant oviposition preference to tomatoes compared to eggplants (Chen et al., 2021). This phenomenon is consistent with the "preference performance hypothesis" (Jaenike, 1978; Thompson, 1988; Mayhew, 1997; Gripenberg et al., 2010). In response to the host plant preference behavior of *T. absoluta*, we supposed that one or more specific plant VOCs released by tomato plants may have attractive effects on *T. absoluta* females, facilitating their rapid localization to tomato plants and preferred oviposition on tomato leaves.

The results from this study showed that 39 differentially accumulated VOCs were identified between the preferred host (tomato) and non-preferred host (eggplant) by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME/GC–MS). Specifically, 15 VOCs were highly accumulated in tomato, with alcohol and heterocyclic compounds the most abundant. These results were different from the previous study, which reported that tomato had a higher number of terpenes and acid compounds than other host plants (including eggplant) (Msisi et al., 2021; Subramani et al., 2021). Our results showed that 24 VOCs were accumulated higher in eggplant with aldehyde and heterocyclic compounds the most abundant. However, some highly accumulated compounds reported in eggplant compared with tomato, such as 1,2,3,5-tetramethylcyclohexane, cyclooctene, 3-(1-methylethenyl), 1,2,3,5-tetramethylcyclohexane, etc. (Subramani et al., 2021), were not found in our study. Similar to previous studies (Proffit et al., 2011; Msisi et al., 2021; Subramani et al., 2021), these differentially accumulated VOCs might contribute to the oviposition preference differences between tomato and eggplant. A previous study reported that the high composition of terpenes in tomato contributed to the attractive activity of tomato volatiles to female *T. absoluta*, while highly constituted green leaves

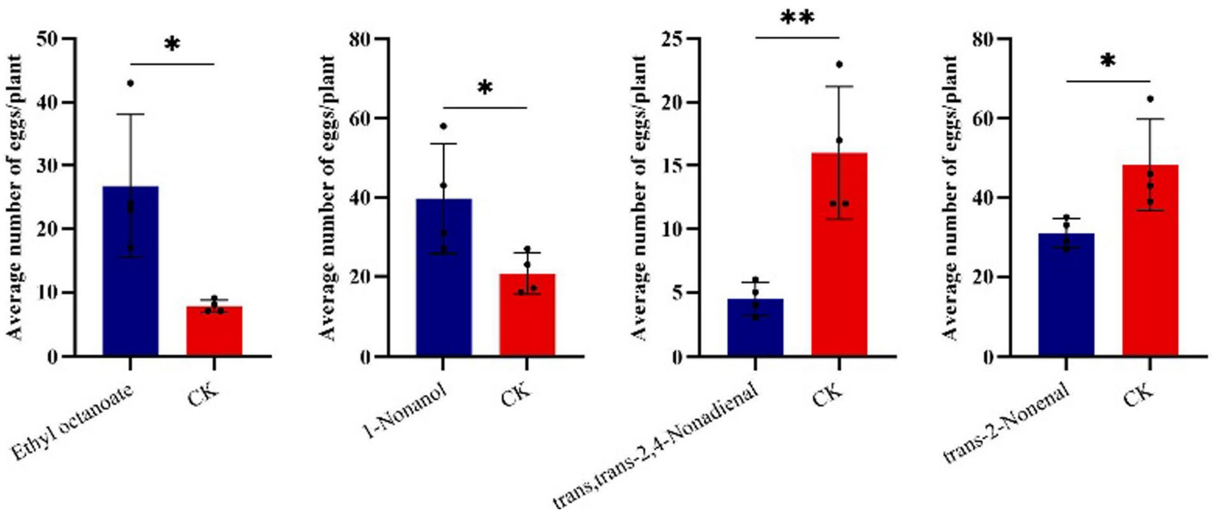

FIGURE 4
The effect of four VOCs on the oviposition behavior of *T. absoluta*. The bar represents the standard error, and asterisks indicate significant differences (*p<0.05; **p<0.01).

volatiles (GLVs) in watermelon contributed to the repellent activity of watermelon volatiles to female *T. absoluta* (Msisi et al., 2021). Subramani et al. reported that volatiles from tomato, such as *p*-quinone, 2-carene, δ-curcumene, and 1,2-diethylbenzene, could serve as oviposition stimulants to *T. absoluta*, whereas the presence of 1-fluorododecane in host plants such as datura, eggplant, ashwagandha, and black nightshade, might deterred *T. absoluta* from ovipositing (Subramani et al., 2021). It has also been reported that plant volatiles from different tomato cultivars contributed to the oviposition choice of *T. absoluta* (Proffit et al., 2011). 2-tridecanone, 2-undecanone, and zingiberene, which are compounds not detected in susceptible tomato varieties, were detected in wild tomato varieties resistant to *T. absoluta* (Leite et al., 1999; Azevedo et al., 2003). Consequently, we suspected the VOCs accumulated higher in tomato might contributed to the higher attraction of tomato plants to *T. absoluta* females for host selection and oviposition, while the VOCs accumulated higher in eggplant might be account for the repellency for egg laying.

To confirm the behavioral effects of the differentially accumulated VOCs on *T. absoluta*, 20 differentially accumulated VOCs had been selected for electroantennographic tests. The results showed 9 VOCs, including 5 highly accumulated in tomato (1-nonanol, 2-phenylethanol, ethyl heptanoate, ethyl octanoate and o-nitrophenol) and 4 highly accumulated in eggplant (2-pentylfuran, trans,trans-2,4-nonadienal, 2-ethyl-5-methylpyrazine and trans-2-nonenal), caused significant higher EAG responses of *T. absoluta* females (Figure 2) and males (Supplementary Figure S1). Further olfactometer bioassays showed that 4 compounds (1-nonanol, ethyl heptanoate, ethyl octanoate and o-nitrophenol) were attractive to *T. absoluta* females, while 5 compounds (2-phenylethanol, 2-pentylfuran, trans,trans-2,4-nonadienal, 2-ethyl-5-methylpyrazine and trans-2-nonenal) were repellent (Figure 3). These results showed that, except for 2-phenylethanol, VOCs that were highly accumulated in tomato elicited attractive activities to *T. absoluta*, while VOCs that were highly accumulated in eggplant elicited repellent activities, and the results for males were highly consistent with those of females (Supplementary Figure S2). These results proved that volatile chemical signals played important roles in the host plant preferences of this pest. The VOCs identified in this study were different from the VOCs with oviposition selection behavior effects on *T. absoluta* in previous studies (Smith et al., 1996; Anastasaki et al., 2018), which could provide new candidate compounds for the development of bisexual attractants and repellents for this pest.

Results from cage experiments confirmed that 1-nonanol and ethyl octanoate were attractive to *T. absoluta* for oviposition. The attractive activities of these two volatiles have been reported in other pests. For instance, 1-nonanol could induce attraction response in sandfly *Lutzomyia longipalpis* (Magalhaes-Junior et al., 2014), melon fly *Bactrocera cucurbitae* (Siderhurst and Jang, 2010), and parasitic wasp *Campsomeris tasmaniensis* (Allsopp, 1992). However, this compound has been reported to be an oviposition deterrent for codling moth, *Cydia pomonella* (Yokoyama and Miller, 1991). Ethyl octanoate itself or synthetic compounds blend containing ethyl octanoate were attractive to fruit flies, such as *Bactrocera dorsalis*, *Anastrepha ludens* and *A. obliqua* (Robacker et al., 1992; Cruz-Lopez et al., 2006; Jayanthi et al., 2012). Ethyl octanoate is also one of the major volatile compounds of fermented sugar baits, which are commonly used for mass trapping of lepidopteran species (El-Sayed et al., 2005). Our results also showed that trans,trans-2,4-nonadienal and trans-2-nonenal could repel *T. absoluta* from oviposition. Trans,trans-2,4-nonadienal has been frequently reported to be a repellent against stored product insects, such as granary weevil *Sitophilus granarius* (Germinara et al., 2015), cigarette beetle *Lasioderma serricorne* and booklouse *Liposcelis bostrychophila* (Wei et al., 2018). Trans-2-nonenal was reported to be repellent to *Culicoides* biting midges (Isberg et al., 2017), and this compound was also effective in inactivating pathogenic bacteria (Cho et al., 2004). Further study should be conducted to test the effects of the identified attractive and repellent VOCs on field populations of *T. absoulta*.

In conclusion, our results identified 39 differentially accumulated VOCs between the preferred host (tomato) and non-preferred host (eggplant). Then the behavioral effects of these VOCs on the host selection and oviposition of *T. absoluta* were further investigated by using electroantennography and olfactometer tests. Almost all the selected VOCs that were highly accumulated in tomato showed attractive activities to *T. absoluta*, while VOCs highly accumulated in eggplants showed repellent activities, indicating that VOCs from host plants play important roles in host plant preferences. The attractive activities of 1-nonanol and ethyl octanoate, as well as the repellent activities of trans,trans-2,4-nonadienal and trans-2-nonenal, were further confirmed in cage experiments. These VOCs will enhance the development of semiochemicals-based eco-friendly control strategies for this pest.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author..

Author contributions

LC and XL: conceptualization and writing—review and editing. TC and YLi: methodology. TC and JW: software. TC, JC, and SY: validation. MH: formal analysis. SZ: investigation. XL: resources. YLi: data curation. YLu: writing—original draft preparation. LC: visualization. TC: supervision. XL: project administration. XL and YLu: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1155317/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Electroantennographic (EAG) responses of *T. absoluta* males to 20 volatile compounds. The bar represents the standard error, and the different letters above each bar indicate Turkey's highest significant difference at $p < 0.05$.

SUPPLEMENTARY FIGURE S2
Responses of male *T. absoluta* to nine VOCs in a Y-type olfactometer. NS indicates no significant difference; asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$).

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EDITED BY
Sadam Hussain,
Northwest A&F University, China

REVIEWED BY
Zhaozhi Lu,
Qingdao Agricultural University, China
Muhammad Hafeez,
Zhejiang University, China
Yongsheng Zhang,
Hunan Agricultural University, China

*CORRESPONDENCE
Xingyuan Men
✉ menxy2000@hotmail.com

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Evaluation of *Osmia excavata* (Hymenoptera: Megachilidae) sensitivity to high-temperature stress

Yingying Song[1], Li Liu[2], Hongying Cui[1], Wenxiu Guo[1], Suhong Lv[1], Baohua Ye[2], Lili Li[1], Yi Yu[1] and Xingyuan Men[1]*

[1]Institute of Plant Protection, Shandong Academy of Agricultural Sciences, Jinan, China, [2]College of Plant Protection, Shandong Agricultural University, Tai'an, Shandong, China

The population of *Osmia excavata*, an important pollinator in commercial orchards, has been in serious decline over recent years. To evaluate the risk of high-temperature stress on *O. excavata*, we evaluated the high-temperature tolerance and potential physiological and biochemical responses of *O. excavata* after a series of high-temperature stresses. The results showed that the mortality rates of *O. excavata* increased gradually with increases in temperature and duration of stress ($R^2 = 0.88–0.99$; $p < 0.05$). The larvae of *O. excavata* were more sensitive to temperature stress than adults, and the median lethal time (LT_{50}) value of the former was smaller than the latter in the acute response test. By comparing the results of acute and chronic responses of *O. excavata* to high-temperature stress, we found that the LT_{50} values of natural cocoon-break adults at slightly higher temperatures (35°C and 40°C) were smaller than those of artificial cocoon-break adults, but the LT_{50} values were similar under extreme high-temperature stress (45°C). Furthermore, the acute and chronic responses on the fat content of adult Osmia obtained by artificial and natural cocoon-break methods were significantly different ($F = 5.03$; $p < 0.05$). Additionally, the mortalities of the young larvae and artificial cocoon-break adults were both significantly and positively correlated with trehalose content ($r = 0.78–0.82$, $p < 0.05$). However, the mortality of the natural cocoon-break adults was negatively related to the acetylcholinesterase activity ($r = –0.93$, $p < 0.001$). Overall, these results suggested that *O. excavata* has a low tolerance to high-temperature stress and provide evidence of causes that could be contributing to the population decline of *O. excavata*.

KEYWORDS
***Osmia excavata*, high-temperature stress, mortality rate, median lethal time, physiological and biochemical index, ecological risk**

Introduction

Osmia excavata (Hymenoptera: Megachilidae) is a univoltine pollinator that spends its entire life inside a bee tube until it emerges the following spring (Men et al., 2018). This insect is used widely to pollinate apple, pear, peach, cherry, and other commonly planted fruit trees in China because of several advantages, including a better pollination efficiency than that of bees and artificial pollination, resistance to low temperature in winter, low take-off temperature (i.e., about 13°C), long daily pollination activity, fast frequency of visiting flowers, and simple feeding and management (Shu et al., 2002; He and Zhou, 2009;

Sgolastra et al., 2015). Known as the king of pollinators (Li, 1992), *O. excavata* has been used for more than 30 years in China (Lu et al., 1992). However, the population of *O. excavata* has been in serious decline in recent years, which has impeded its function in ecological pollination (Cao et al., 2017; Liu et al., 2018). Many studies have found that the lethal factors in the decline of *O. excavata* have included residual pesticide in pollen, egg abortion, predation by parasitic mite wasps, and natural death (Yu, 1999; Zhai et al., 2016; Song et al., 2021), while Liu et al. (2018) believed that natural death was the main cause among the other factors. Cao et al. (2017) also reported that nearly 80% of *O. excavata* would die due to unsuitable growing conditions. However, relevant research is still very limited.

According to investigations, farmers in China usually hang the collected bee tubes with *O. excavata* in a ventilated outdoor environment, and occasionally put them under the eaves (Yu, 2014). However, this method of storing *O. excavata* can increase the risk of high temperatures in the following ways. First, the temperature outside is approximately 3.8°C higher than that in the ambient conditions (Zhou et al., 2018). Second, an outdoor storage environment is vulnerable to short periods of direct sunlight, which in turn creates a greenhouse effect inside the cocoon. Finally, global warming is already causing extreme high temperatures and heat waves in many parts of the world, and this phenomenon is posing a serious threat to biodiversity (García-Robledo et al., 2016; IPCC, 2021). It is reported that high-temperature events have increased by 40% over the past 60 years and the duration, frequency, and intensity of heat waves are predicted to increase with a 90%–99% probability (Ju et al., 2013).

Although insects are ectotherms, the growth and development of insects including *O. excavata* need to be carried out in an optimal temperature range (Zhang et al., 2020; Kuczyk et al., 2021). Otherwise, the fitness of the insect would be severely decreased. Chen et al. (2018) reported that the lethal temperatures for most insects are usually between 40°C and 50°C, depending on the species and life stage. Some insects are even at risk of extinction at current projected rates of global warming (García-Robledo et al., 2016; Abou-Shaara et al., 2017; Wang et al., 2017). For example, when the growth temperatures are higher than 36°C, a colony of honey bees is likely to be exposed to superheated temperatures, which would impact the adult brain (Abou-Shaara et al., 2017); short-term high-temperature stress can also decrease oviposition in *Bactrocera cucurbitae* and *Carposina sasakii* (Zeng et al., 2019; Zhang et al., 2020), and increase the instantaneous death risk of *Ostrinia furnacalis* (Zhou et al., 2018). However, few relevant studies on how *O. excavata* respond to high-temperature stress exist due to their long life history and the difficulty in observing their development status in the cocoon.

Thus, the objective of this research was to evaluate the high-temperature tolerance and potential physiological and biochemical responses of *O. excavata* after a series of high-temperature stresses. We speculated that (i) the higher is the stress temperature and longer the stress period, the greater the risk of *O. excavata* mortality; (ii) the acute and chronic responses of *O. excavata* to high-temperature stress may be different and; (iii) the physiological and biochemical substances of *O. excavata* and resisting high-temperature stress may be disturbed under high-temperature stress.

Materials and methods

Insects

The population of *O. excavata* was commercial, and acquired from Yantai Bifeng Agricultural Technology Co. Ltd., China. It has been continuously mass-reared for more than 10 generations in fruit orchards in Shandong Province, China. No pesticides were sprayed 20 days before flowering and throughout the flowering period.

High-temperature stress

The effects of high-temperature stress on *O. excavata* refer to previous research methods with minor modifications (Wang et al., 2017; Zhang et al., 2020). The tubes containing *O. excavata* were placed in growth climatic chambers (RXZ-600C, Ningbo Jiangnan, China) at different temperatures (30°C, 35°C, 40°C, and 45°C) during the young (2nd instar) larval, mature (5th instar) larval, and adult stages of *O. excavata*. After a certain period of temperature stress treatment, the larval and adult of *O. excavata* were taken out for life index detection.

Measurement of mortality

In the acute response test, the young and mature larvae and artificial cocoon-break adults of *O. excavata* were measured. Bee tubes were opened by hand and cocoons were artificially and carefully dissected with scissors after different periods of high-temperature stress and the mortality of *O. excavata* was observed according to the method of Song et al. (2021). There were three tubes per replicate (i.e., 18–24 larvae or cocoons per replicate) and three repeats (i.e., 54–72 larvae or cocoons per treatment) every sample date. The number of cocoons in each tube (6–8 cocoons per tube) was determined according to the egg-laying situation of maternal *O. excavata*. Mortality was observed at 0.25 h intervals, the longest observation period was 2.5 h for larvae and 24 h for adults.

To observe the long-term effects of high-temperature stress on the adults, the chronic response for natural cocoon-break adults was tested. The cocoons containing *O. excavata* that have been exposed to heat stress for a certain length of time were transferred to their optimal growing environment, that is a darkroom with 65%–75% relative humidity and 25°C ± 2°C temperature (Song et al., 2021). Then the mortality of *O. excavata* was observed after a natural cocoon break.

Physiological and biochemical indexes

The young and mature larvae, natural cocoon-break, and artificial cocoon-break adults of *O. excavata* were both used to measure the physiological and biochemical indexes after high-temperature stress exposure. There were three tubes per replicate and three repeats.

The trehalose content in each *O. excavata* was assayed using a trehalose quantification kit (Suzhou Keming Biotechnology Co., Ltd., China). In brief, each *O. excavata* was weighted using an electronic balance (AL104; Mettler-Toledo, China). The lapping liquid of *O. excavata* and extracting solution was left standing at room temperature for 45 min and oscillated 3–5 times. After cooling, the sample was centrifuged for 10 min at 8,000 rpm and 25°C to obtain the supernatant. The value was recorded spectrophotometrically at 620 nm.

Acetylcholinesterase (AchE) activity was measured using a kit provided by Suzhou Keming Biotechnology Co., Ltd. The *O. excavata* and extracting solution were ground to homogenate under ice bath conditions. The abrasive liquid was centrifuged for 10 min at 8,000 rpm and 4°C. Then, the activity of AchE was evaluated spectrophotometrically at 412 nm.

The fat and free water were also assayed. First, the fresh weight of *O. excavata* was determined using an electronic balance. Then the body was dried for 48 h at 60°C and the dry weight was determined. The free water was obtained by calculating the difference between the wet and dry weight of *O. excavata*. Then dried *O. excavata* was added to 2 ml of chloroform:methanol (2:1), and fully ground until homogenized. Afterward, the homogenate was centrifuged for 10 min at 2,600 rpm and the supernatant was discarded. Another 2 mL of the mixture was added to the residue, the centrifugation was repeated once, and the supernatant was discarded. The remaining residue was dried for 72 h at 60°C to constant weight. Finally, the fat was calculated according to a method described previously (Colinet et al., 2007).

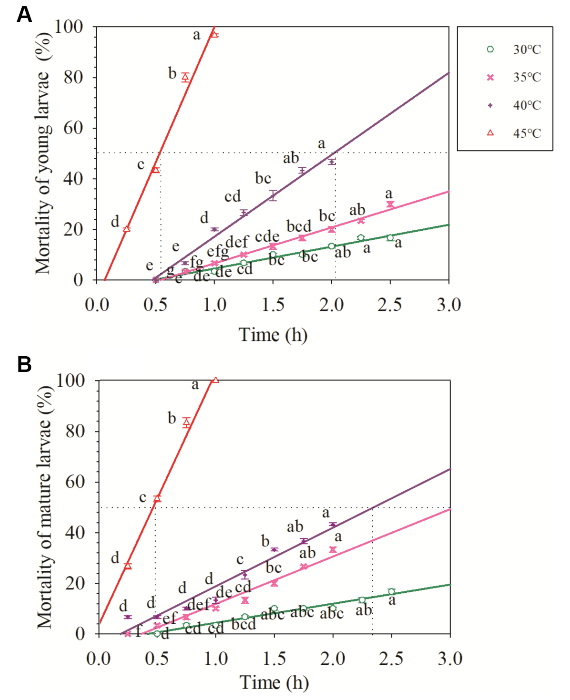


FIGURE 1
Acute response of the young and mature larvae of *Osmia excavata* to different durations of high-temperature stress (different lowercase letters indicate significant differences between different stress times at the same temperature according to Tukey's LSD test at p <0.05).

Data analysis

Before statistical analysis, data were transformed to $\log_{10}$, arcsine, or square-root when necessary to evaluate data normality and homogeneity. The median lethal time (LT_{50}) and analysis of variance were determined using SPSS v.21.0 (SPSS Inc., Chicago, IL; Abbott, 1925). One-way analysis of variance (ANOVA) was used to estimate the impact of different duration of high-temperature stress on the mortality and the physiological and biochemical indexes of *O. excavata*. Two-way ANOVAs were performed to determine the impact of insect stages (or response mode) and high-temperature stress on the physiological and biochemical indexes of *O. excavata*. Means were compared by using Tukey's LSD test at $p < 0.05$. Pearson's correlation was used to analyze the relationships between the mortality and the physiological and biochemical indexes of *O. excavata* at different insect stages under high-temperature stress.

Results

Acute responses of *Osmia excavata* to high-temperature stress

The mortality of *O. excavata* significantly increased with an increase in the duration of high-temperature stress ($p < 0.05$; Figure 1) and increased with an increase in temperature ($R^2 = 0.92–0.99$; Table 1).

For the young larvae of *O. excavata,* the mortality at 30°C for 2.5 h was about 15%, and that at 35°C for 2.5 h was about 30%, but those were significantly higher than those at 30°C and 35°C for less than 1.75 h (+66.7% and +79.96%), respectively ($p < 0.05$). The mortality of young larvae at 40°C for 1.75–2.0 h was more than 40%, and both were markedly higher than those at 40°C for less than 1.25 h ($p < 0.05$). The mortality of the young larvae at 45°C for 1.0 h reached 96.67%, and significantly higher than that at 45°C for 0.75 h (+20.84%; $p < 0.05$). The young larvae did not die when the temperature was lower than 40°C for 0.5 h, but the mortality reached 20% after 0.25 h at 45°C (Figure 1). Additionally, the LT_{50} of the young larvae decreased with an increase in temperature (Table 1). The LT_{50} of the young larvae at 30°C and 35°C were over 3 h, and at 40°C and 45°C, they were 2.01 and 0.53 h, respectively ($R^2 = 0.98–0.99$; Table 1; Figure 1).

For the mature larvae of *O. excavata*, the mortality at 30°C for 2.25 h was about 13% and significantly higher than that at 30°C for less than 1.25 h (+100%; $p < 0.05$). The mortalities of the mature larvae at 35°C and 40°C for 2.0 h were markedly higher than those for 1.5 h, respectively (+66.65% and + 30%; $p < 0.05$). The mortality of the mature larvae at 45°C for 1.0 h reached 100%, and significantly higher than that at 45°C for 0.75 h (+20%; $p < 0.05$; Figure 1). The LT_{50} of the mature larvae at 40°C and 45°C were 2.35 h and 0.47 h, respectively ($R^2 = 0.95–0.99$; Table 1; Figure 1).

For the artificial cocoon-break adults, the mortality increased slowly with time at 35°C and 40°C, but rapidly at 45°C (Figure 2). The mortality of the artificial cocoon-break adults at 45°C for 0.5 h was nearly 50% and markedly increased to 90% after 2 h (+107.14%; $p < 0.05$). The LT_{50} of the artificial cocoon-break adults at 40°C and 45°C were more than 25 and 0.6 h, respectively ($R^2 = 0.92–0.99$; Table 1; Figure 2).

TABLE 1 Effects of high-temperature stress on the time of death in *Osmia excavata* at different stages of development, including young larvae, mature larvae, artificial cocoon-break adults, and natural cocoon-break adults.

Response	Insect stage	Temperature (°C)	Regression equation	LT_{50} (h)	R^2
Acute response	Young larvae	30	$y = 8.67x - 4.11$	>3	0.98
		35	$y = 14.22x - 7.63$	>3	0.99
		40	$y = 32.38x - 15.24$	2.01	0.98
		45	$y = 106.67x - 6.67$	0.53	0.98
	Mature larvae	30	$y = 7.56x - 3.19$	>3	0.95
		35	$y = 18.73x - 6.90$	3.04	0.97
		40	$y = 23.18x - 4.40$	2.35	0.95
		45	$y = 100x + 3.33$	0.47	0.99
	Artificial cocoon-break adults	35	$y = -0.02x^2 + 1.04x + 2.31$	–	0.92
		40	$y = 0.01x^2 + 0.78x + 14.40$	>25	0.97
		45	$y = -7.61x^2 + 48.18x + 24.01$	0.6	0.99
Chronic response	Natural cocoon-break adults	35	$y = 0.18x^2 - 2.53x - 9.62$	>25	0.95
		40	$y = -0.17x^2 + 7.02x - 18.06$	15.55	0.88
		45	$y = -10.48x^2 + 70.95x - 33.34$	1.51	0.99

LT_{50}, median lethal time.

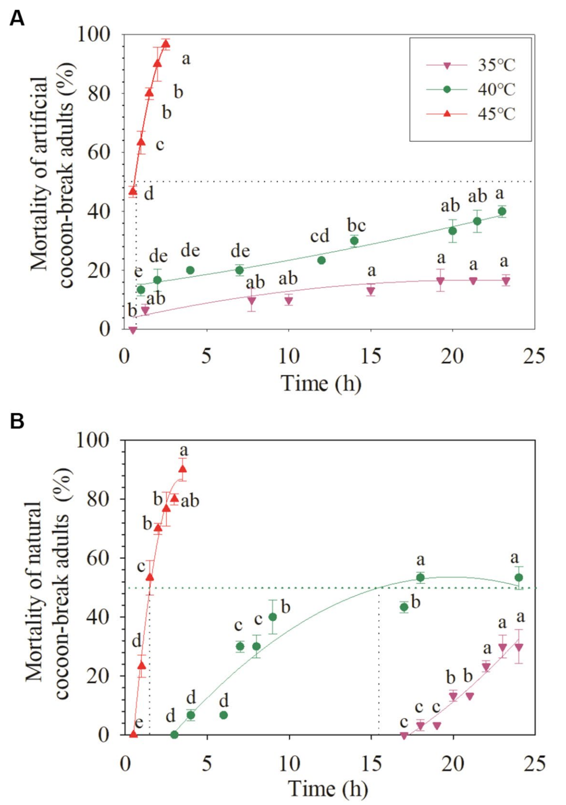


FIGURE 2
Acute response of the artificial cocoon-break adults and chronic response of the cocoon-break adults of *Osmia excavata* to different durations of high-temperature stress (different lowercase letters indicate significant differences between different stress times at the same temperature according to Tukey's LSD test at $p < 0.05$).

Chronic responses of *Osmia excavata* to high-temperature stress

In the natural cocoon-break adults of *O. excavata*, no death occurred under 35°C stress after 17 h, but the mortality increased significantly to 30% after 24 h ($p < 0.05$; Figure 2). The mortality of natural cocoon-break adults increased faster with time at 40°C than that at 35°C, but slower than that at 45°C ($p < 0.05$). The mortality of natural cocoon-break adults at 45°C for 1 h was nearly 23.33% and markedly increased to 90% after 3.5 h at 45°C (+285.77%; $p < 0.05$). The LT_{50} of natural cocoon-break adults at 40°C and 45°C were 15.55 and 1.51 h, respectively ($R^2 = 0.88–0.99$; Table 1; Figure 2).

Physiological and biochemical indexes of *Osmia excavata* under high-temperature stress

Although the acute response of the physiological and biochemical indexes of *O. excavata* were significantly different between young larvae, mature larvae, and artificial cocoon-break adult stages ($F = 5.52–653.19$; $p < 0.01$), the high-temperature stress had no significant effect on the acute response of the physiological and biochemical indexes ($F = 0.36–0.88$; $p > 0.05$; Table 2). There were significant interactions of the fat content between temperature stress and insect stage ($F = 3.38$; $p < 0.01$). Additionally, the trehalose content of the mature larvae at 35°C was significantly increased compared with that at 30°C (+126.20%; $p < 0.05$; Figure 3). The fat content of the artificial cocoon-break adults at 45°C was markedly higher than that at 35°C (+32.12%; $p < 0.05$). The free water content was significantly decreased compared with that at 35°C (−9.21%; $p < 0.05$; Figure 3).

There was no significant difference between the acute and chronic responses on the physiological and biochemical indexes of adult

TABLE 2 Effects of high-temperature stress on the acute response of the physiological and biochemical indexes of *Osmia excavata* at different stages of development, including young larvae, mature larvae, and artificial cocoon-break adults (*F* value).

Impact factors	*df*	Trehalose (mg/mg prot)	Acetylcholin esterase (nmol/min/mg prot)	Fat (%)	Free water (%)
Temperature stress (T)	3	0.88	0.36	0.59	0.51
Insect stage (I)	2	5.52**	11.12***	653.19***	123.45***
T × I	5	1.13	1.45	3.38 **	1.10

**$p < 0.01$; ***$p < 0.001$.

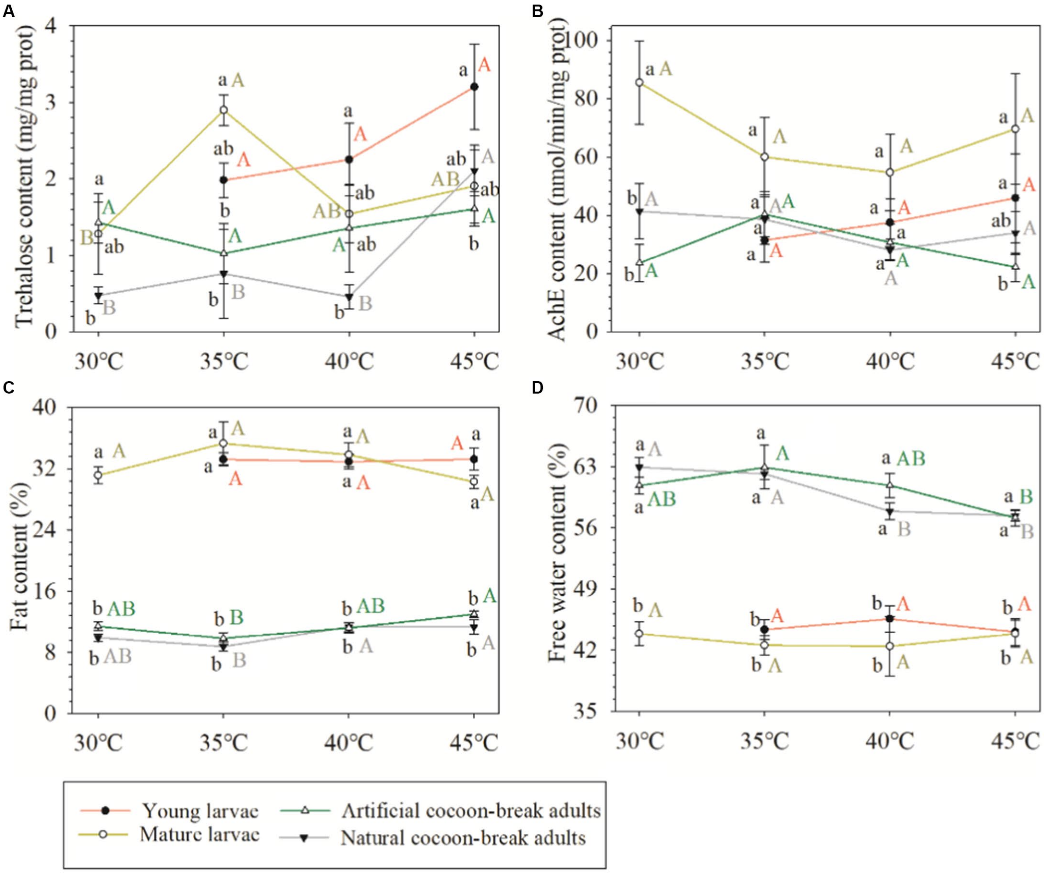


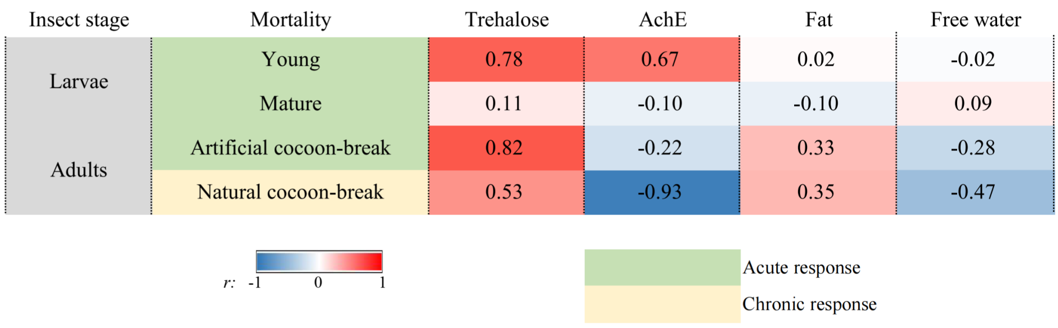
FIGURE 3
Effects of high-temperature stress on the physiological and biochemical indexes of *Osmia excavata* at different stages of development, including young larvae, mature larvae, artificial cocoon-break adults, and natural cocoon-break adults (AchE, acetylcholinesterase; different lowercase letters indicate significant differences between different insect stages at the same temperature stresses according to Tukey's LSD test at $p < 0.05$; different capital letters indicate significant differences between different temperature stresses in the same insect stage at $p < 0.05$).

TABLE 3 Physiological and biochemical indexes of the acute and chronic responses of adult Osmia obtained by artificial and natural cocoon-break methods to high-temperature stress (*F* value).

Impact factors	*df*	Trehalose (mg/mg prot)	Acetylcholin esterase (nmol/min/mg prot)	Fat (%)	Free water (%)
Temperature stress (T)	3	4.38**	0.56	6.86***	116.18
Response mode (R)	1	2.76	1.42	5.03*	2.03
T × R	3	2.36	1.2	0.78	21.46

Response mode, acute and chronic responses of adult Osmia; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Osmia obtained by either artificial or natural cocoon-break methods ($F = 1.42–2.76$; $p > 0.05$), except the fat contents ($F = 5.03$; $p < 0.05$; Table 3). The high-temperature stress had a significant effect on the contents of trehalose and fat ($F = 4.38–6.86$; $p < 0.05$). The trehalose content of adult Osmia obtained by the natural cocoon-break method at 45°C was significantly higher than that at 30°C (+359.31%;



Insect stage	Mortality	Trehalose	AchE	Fat	Free water
Larvae	Young	0.78	0.67	0.02	-0.02
	Mature	0.11	-0.10	-0.10	0.09
Adults	Artificial cocoon-break	0.82	-0.22	0.33	-0.28
	Natural cocoon-break	0.53	-0.93	0.35	-0.47

FIGURE 4
Pearson's correlation between the mortality and the physiological and biochemical indexes of the young larvae, mature larvae, artificial cocoon-break adults, and natural cocoon-break adults of *Osmia excavata* under high-temperature stress (*r* value). The darker the blue or red, the stronger the negative or positive correlation, respectively.

$p < 0.05$), 35°C (+177.17%; $p < 0.05$), and 40°C (+342.15%; $p < 0.05$). The fat content of adults by the natural cocoon-break method at 45°C was markedly higher than that at 35°C (+29.59%; $p < 0.05$; Figure 3).

Correlation analysis between the mortality and physiological and biochemical indexes of *Osmia excavata*

The mortality of the young larvae was significantly and positively correlated with the trehalose content ($r = 0.78$, $p < 0.05$) and AchE activity ($r = 0.67$, $p < 0.05$; Figure 4). The mortality of the artificial cocoon-break adults of *O. excavata* was also significantly and positively related to the trehalose content ($r = 0.82$, $p < 0.01$). However, the mortality of the natural cocoon-break adults was negatively related to the AchE activity ($r = -0.93$, $p < 0.001$). The mortality of the mature larvae was not significantly correlated with physiological and biochemical indexes [$r = (-0.10) - 0.11$, $p > 0.05$; Figure 4].

Discussion

High-temperature stress poses a serious risk to *Osmia excavata*

Temperature is one of the most important external conditions affecting the life activities of insects (Dongmo et al., 2021; Gaytán et al., 2022), but an abnormal high-temperature environment has a serious impact on growth and development, and even causes the risk of extinction in some insects (García-Robledo et al., 2016; Abou-Shaara et al., 2017; Wang et al., 2017). Populations of *O. excavata* are more easily affected by adverse factors because they only have one generation a year (Men et al., 2018). Our results demonstrated that the higher the temperature, the faster the death rate of *O. excavata*, which was consistent with our hypothesis (i). Similar results have been obtained in *Carposina nipponensis* (Zhang et al., 2020), *Ophraella communa* (Chen et al., 2018), and *Corythucha ciliata* (Ju et al., 2013). In addition, this study found that under a slightly higher temperature (35°C and 40°C), the LT_{50} value of adults was higher than that of larvae. Thus, we speculated that the harmful effects of high temperatures on *O. excavata* depend not only on the intensity and duration of stress but also on the developmental stage (Enriquez and Colinet, 2017). As the adults of *O. excavata* will naturally emerge from the cocoon with the rising temperature in the spring (Men et al., 2018), this may be the reason for a higher temperature tolerance in adults than in larvae. By comparing the results of acute and chronic responses of *O. excavata* to high-temperature stress, it was found that the LT_{50} values of natural cocoon-break adults at 35°C and 40°C were smaller than those of artificial cocoon-break adults, but similar LT_{50} values were observed at 45°C. This may occur for three reasons: (a) the physiological and biochemical substances that maintain the normal growth and development of *O. excavata* were destroyed after slightly higher temperature stress, even if they did not die immediately, but it was not enough to support their survival for long; (b) the functions related to cocoon breaking were severely damaged and failed to successfully break the cocoon; (c) extreme high-temperature stress (45°C) may cause *O. excavata* to die instantly. Considering the high mortality we found in larvae and adult Osmia, apiarists should store bee tubes in a cool ventilated place out of direct sunlight to maintain an adequate population of *O. excavata*.

Metabolites in larvae of *Osmia excavata* are more susceptible to high temperature stress than those in adults

Trehalose is an important blood sugar in insects, not only can it be stored as an energy source and carbohydrate reserve but also as a compatible solute adapted to various stresses, such as heat, cold, osmotic stress, and drought (Xin et al., 2013; Qin et al., 2015). There was no significant difference in the trehalose content in larvae and artificial cocoon-break adults at 30°C, but the trehalose content in the former increased faster than the latter with an increase in temperature, especially when the temperature reached 40°C and 45°C, the content and growth rate of trehalose in the young larvae were both higher than that of the mature larvae. These results showed that the smaller the insect stage under high-temperature stress, the more drastic the change of trehalose content in *O. excavata*. This may be due to the poor ability of the younger larvae to resist an adverse environment. Jiang et al. (2016) also believed that the body surface of younger larvae was soft and its cuticle was thin, but the body surface of adult insects was relatively hard and the cuticle layer was thicker, which can reduce stress and retain normal life activities of adults.

Fat plays an important role in the energy storage and metabolism of insects (Wu et al., 2019). At the same time, insects cannot live without the participation of free water, which is a good solvent in the cell, can participate in the cellular biochemical reaction, and transport nutrients and waste produced by metabolism. Adults of *O. excavata* were found to have less fat and more free water than larvae. The temperature in the larval stage of *O. excavata* is relatively low under natural conditions, so the larvae would resist the cold climate by storing more fat and reducing free water content; as temperatures rise in the adult stage, the *O. excavata* would break diapause, cocoon breaking, flight, etc., therefore, they need to use more energy and free water to speed up metabolism (Sgolastra et al., 2015; Men et al., 2018). It was also found that the contents of fat and free water in larvae were not significantly affected after extreme heat stress, whereas the contents in adults were more affected, indicating that the fat and free water contents in larvae of *O. excavata* were less sensitive to high-temperature stress than that in adults. This may be because the larvae of *O. excavata* have fewer activities and slower metabolism than adults in the cocoon, but further research is needed.

Special metabolism in adult *Osmia excavata* after high-temperature stress

In the chronic response test, the trehalose content of adult Osmia was significantly increased after extreme high-temperature stress compared to slightly higher temperature stress. We speculated that there may be two reasons: (a) trehalase was inactivated in insects under extreme high-temperature stress, so trehalose could not be hydrolyzed, and more trehalose content was accumulated (Qin et al., 2015); (b) a special protective membrane is formed on the cell surface by increasing trehalose content under harsh conditions, such as extreme high temperature, to prevent the structure of biomolecules from being disrupted (Ma et al., 2018). These results were similar to previous studies on *Monolepta hieroglyphica* and *Gomphocerus sibiricus* and suggested that this was a manifestation of insect adaptation to high-temperature stress (Li et al., 2014; Ma et al., 2018). However, in the acute response test, the trehalose contents of larvae or artificial cocoon-break adults were not markedly changed after being subjected to high temperatures. We believed that although the trehalose content in *O. excavata* increased with an increase in temperature stress at each insect stage, it did not increase to a significant level in a short time.

The fat contents in natural and artificial cocoon-break adults of *O. excavata* after extreme high-temperature stress were both significantly higher than those after slightly high-temperature stress, but the change in the trend of free water was opposite to that of the fat content. Zhao et al. (2010) found that low-temperature stress can also lead to an increase in insect fat content. We believe that the reasons for the increase in fat content are different between high and low-temperature stress: the former may be due to the serious loss of free water in the adult body of *O. excavata* under extreme temperature stress, which hinders normal metabolism and inhibits the decomposition of fat, even causing adults to die faster; the latter may be because insects would reduce their supercooling point in a low-temperature environment by increasing their fat content, which can increase their cold tolerance and resist cold damage (Zhao et al., 2010). Interestingly, Liu et al. (2010) reported that heat-shock stress decreased lipid storage in planarian *Dugesia japonica* to suppress its development. We also found that the fat content of adults decreased at 35°C compared with 30°C, but did not reach a significant level. This may be because the life activities of *O. excavata* have not been completely disordered after they endured tolerable temperature stress, and the metabolic ability was enhanced to maintain normal life activities by consuming more energy substances such as fat.

Conclusion

To date, the population of *O. excavata* has been in serious decline and there is a lack of observation on the sensitivity of *O. excavata* to high-temperature stress. The current study found that the mortality of *O. excavata* significantly increased with the increased temperature and duration of high-temperature. Additionally, the larvae of *O. excavata* were more sensitive to the same temperature stress than adults, and the LT_{50} value of the former was smaller. Furthermore, there was a significant difference between the acute and chronic responses on the fat content of adult Osmia by both the artificial and natural cocoon-break methods. And the mortalities of the young larvae and artificial cocoon-break adults were both significantly and positively correlated with the trehalose content. Given the potential impacts of high-temperature stress on the cocoon break, flight ability, and fertility of *O. excavata*, the adverse impact of temperature on the population of *O. excavata* needs further study.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

YS, YY, BY, and LLiu participated in the experimental investigation and analyzed the data. HC, WG, SL, and LLi contributed reagents/materials. YS wrote the first draft of the article. XM revised the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Fang Ouyang,
Chinese Academy of Sciences (CAS), China

REVIEWED BY
Hongying Cui,
Shandong Academy of Agricultural Sciences, China
Yingying Song,
Shandong Academy of Agricultural Sciences, China

*CORRESPONDENCE
José Neiva Mesquita-Neto
✉ jmesquita@ucm.cl

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Pollination by native bees achieves high fruit quantity and quality of highbush blueberry: a sustainable alternative to managed pollinators

Benito Cortés-Rivas[1,2], Víctor Hugo Monzón[2], Juliana Ordones Rego[2] and José Neiva Mesquita-Neto[2]*

[1]Facultad de Ciencias Forestales y Recursos Naturales, Instituto de Biodiversidad, Conservación y Territorio, Universidad Austral de Chile, Valdivia, Chile, [2]Laboratorio Ecología de Abejas, Departamento de Biología y Química, Facultad de Ciencias Básicas, Universidad Católica del Maule, Talca, Chile

Introduction: Blueberry is one of the most relevant buzz-pollinated crops worldwide, and Chile is the most important global producer of fresh blueberries during wintertime in the Northern Hemisphere. Non-buzzing bees, such as honey bees, may provide suboptimal services compared with bees capable of buzz pollination. The widely held contention that honey bees are inferior pollinators of blueberries drives the industry to place pressure on governments to allow bumblebee (*Bombus terrestris*) importation for pollination. However, the introduction of *B. terrestris* generates environmental problems in Chile by competing with and transmitting parasites to local bees. Despite some native Chilean bees being recently recognized as efficient pollen vectors of blueberry crops, no study has evidenced the influence of their visits on fruit yield. Therefore, we aimed to evaluate the native Chilean floral visitors' performance to improve the quantity and quality of highbush blueberry in comparison to the performance of managed visitors.

Methods: Per-visit pollination performance (fruit set and fruit quality) and visitation frequency were measured, and the performance of buzzing behavior by flower visitors was evaluated in four cultivars grown in five blueberry orchards located in southern Chile.

Results: We found that fruit set and weight were highly influenced by floral visitor taxon. Some native bee species can greatly improve the fruit set and fruit quality (greater weight) of the highbush blueberry cultivars. For instance, one single visit of *C. occidentalis* can increase fruit weight by a factor of 1.8 compared to an *A. mellifera* visit; however, visits of halictids and syrphids resulted in lower fruit sets than those of unvisited flowers. However, we found that the occurrence of sonication behavior alone was not a predictor of higher fruit set and fruit weight of highbush blueberry cultivars. Consequently, the taxonomic recognition of floral visitors, ideally to the species level, is still needed to distinguish the most efficient fruit yield promoters of blueberry.

Discussion: The conservation of the biotic pollinators, especially native pollinators, would improve blueberry fruit quality and is likely to improve overall crop productivity.

KEYWORDS

buzz pollination, buzz pollinated crops, Chile, ecosystem services, non-apis bees, *Vaccinium corymbosum*

1. Introduction

World blueberry production has more than doubled in the last 10 years, reaching 823,328 tons in 2019. Of this, 58% is produced in North America, with38% in the United States and 20% in Canada (FAO, 2020). Following the same trend, the area of Chilean blueberry cultivation has increased rapidly during the last 30 years (Retamales and Hancock, 2018). In 2021, Chile had 18,000 hectares planted with blueberry, exported 118,225 tons of fresh blueberry fruit, and was the third greatest provider of fresh blueberry fruit to the Northern Hemisphere during its winter (Velasco, 2021). However, there are several challenges to producing blueberry fruits of high and consistent quality (Retamales and Hancock, 2018). The number of fruits per plant and their fresh weight are two fundamental factors of blueberry plant yield (Hall et al., 2020). Large and consistent berries increase the market price (Gilbert et al., 2014).

High-quality fruit production by wild and commercial blueberries is highly dependent on the input of biotic pollinators (MacKenzie and Eickwort, 1996; Fulton et al., 2015). Studies in the native range of southern and northern highbush blueberries reveal that the most effective pollinators of blueberries are typically large bees that can extract pollen from anthers by vibrating their bodies during floral visits (Stubbs and Drummond, 1996; Javorek et al., 2002; Campbell et al., 2018). The vibrations produce an audible buzzing sound, which gives the name to this pollination syndrome: buzz pollination or floral sonication (Vallejo-Marín, 2019; Pritchard and Vallejo-Marín, 2020).

The predominant floral trait related to buzzing behavior is the presence of poricidal anthers (Buchmann, 1983; De Luca and Vallejo-Marin, 2013). For flowers with poricidal anther dehiscence, the only exit of pollen from the flower is via tiny openings at the apex of the anthers (Delaplane et al., 2000). This is the case for blueberry flowers and flowers of more than 20,000 species of native and cultivated flowering plants (Buchmann, 1983). Although most buzz-pollinated flowers are nectar-less (Vallejo-Marín et al., 2010), in some cases, such as with blueberry, flowers also produce floral nectar (Javorek et al., 2002), which implies that even a flower visitor capable of sonicating will not necessarily do so if it is looking for nectar. Furthermore, although hives of the honey bee (*Apis mellifera*) are widely managed for blueberry pollination throughout the world (Benjamin et al., 2014; FAO, 2020), these bees are unable to sonicate and tend to present lower per-visit pollen deposition rates than bees capable of vibrating flowers (Javorek et al., 2002; Rogers et al., 2013; Cortés-Rivas et al., 2023). Thus, honey bees and other non-buzzing bees may provide a suboptimal service compared with other bees capable of buzz pollination (Stubbs and Drummond, 1996; Javorek et al., 2002; Kim et al., 2005; Campbell et al., 2018; Nicholson and Ricketts, 2019; Cooley and Vallejo-Marín, 2021).

The widely held contention that honey bees are inferior pollinators of blueberries drives the industry to place pressure on governments to allow bumblebee (*Bombus terrestris*) importation for pollination. Managed beehives of *B. terrestris* achieve high yields due, partially, to their ability to sonicate flowers and be active during environmental conditions unfavorable to honey bees (De Luca et al., 2013; Howlett et al., 2019; Cortés-Rivas et al., 2023). However, the introduction of *B. terrestris* generates serious problems for the conservation of native pollinators since this species is highly invasive where it is introduced and can compete and transmit parasites to native bees (Matsumura et al., 2004; Dafni et al., 2010; Morales et al., 2013; Smith-Ramírez et al., 2018). As a result, strict laws prohibiting the movement of bumblebees are in place in parts of the USA (Winter et al., 2006), southern Africa, and Australasia (Goka, 2010; Inoue and Yokoyama, 2010) because their introduction could have undesired effects on native fauna and flora (Velthuis and Van Doorn, 2006; Goka, 2010; Inoue and Yokoyama, 2010).

However, the performance of native fauna to pollinate blueberry crops in Chile has recently been revealed, with some bee species having high conspecific pollen transference (Cortés-Rivas et al., 2023). These highly efficient native species would be a more sustainable alternative to *B. terrestris* importation. Despite this encouraging finding, no study has evidenced the influence of the visits of these native species on fruit yield. Therefore, distinguishing the pollinators associated with higher fruit quantity and quality among the local pool of visitor species is essential to reveal their relative importance for agricultural production (Javorek et al., 2002; Isaacs and Kirk, 2010; Nicholson and Ricketts, 2019). Thus, we aimed to evaluate the native Chilean floral visitors' ability to improve highbush blueberry fruit yield compared to that of managed visitors. We expected that, due to the capacity of some native bee taxa to perform sonication and transfer a high amount of conspecific pollen, their visits would result in higher fruit set and fruit weight in relation to *A. mellifera* and at least similar to *B. terrestris*, making them suitable alternative candidates to improve fruit yield of blueberry crops in Chile (Hypothesis 1). Because the occurrence of floral sonication is a predictor of higher pollen deposition on the stigmatic surface of blueberry flowers (Cortés-Rivas et al., 2023), we wanted to know if buzzing behavior was associated with higher fruit quantity and/or quality, so we compared blueberry fruit production between the subset of visits with floral sonication and the subset of visits without this behavior. We chose this approach to focus on the influence of the buzzing behavior itself rather than having to assume that a bee belonging to a taxon capable of vibrating does necessarily vibrate on every visit. We expected that visits to highbush blueberry flowers accompanied by buzzing behavior would be associated with higher fruit set, seed set, and fruit weight than visits without this behavior (Hypothesis 2).

2. Materials and methods

2.1. Study areas

Fieldwork was conducted in five highbush blueberry orchards located in southern Chile (Maule and Los Ríos Regions; Supplementary Figure S1; Table 1) between September and November in 2020 and 2021. The total area of cultivated blueberries per orchard ranged from 3.2 to 141 hectares of both organic and conventional farming. The most common growing cultivars were Legacy, Brigitta, Duke, Elliot, and Draper. Four of the five orchards were supplemented with colonies of managed exotic bees of *Bombus terrestris* and/or *Apis mellifera* (Table 1). The

TABLE 1 Highbush blueberry orchards located in the southern region of Chile (Maule and Los Ríos Regions), where this study was conducted from September to November of 2020 and 2021.

Orchard	Locality/region	Latitude, longitude	Farming type	Area	Cultivars	Managed bee(s)
Agrícola Aguas Negras	Paillaco/Los Ríos	40°2'55.62"S, 72°45'15.20"W	Conventional	28 ha	Brigitta, Legacy, Elliot, Draper, Duke	*Bombus terrestris/Apis mellifera*
Shine Liucura	Paillaco/Los Ríos	40°2'49.89"S, 72°46'49.21"W	Organic	8.1 ha	Brigitta, Bluecrop, Coville, Elliot, Legacy	*Bombus terrestris*
Agroberries Asque	Mariquina/Los Ríos	39°33'59.4"S, 72°59'28.4"W	Organic	141 ha	Brigitta, Duke, Elliot, Legacy, Topshelf	*Apis mellifera*
Agroberries Cun Cun	Mariquina/Los Ríos	39°33'44.0"S, 73°02'33.8"W	Conventional	114 ha	Brigitta, Duke, Elliot, Legacy, Topshelf	*Apis mellifera*
Agrícola Campos Álvarez	Linares/Maule	35°55'45.8"S, 71°29'37.9"W	Conventional	3.2 ha	Duke, Legacy.	None

Information about the geographical location, farming type, cultivated area, blueberry cultivars, and bee species managed for crop pollination.

local temperature varied between 10°C and 37°C (average 20.2°C), and the wind varied between 0.16 and 8.85 km/h.

2.2. Pollination efficiency and fruit yield

We measured fruit set and seed set based on the single visit test (Ne'eman et al., 2010). To conduct this test, we randomly selected and labeled 10 individual plants per blueberry cultivar/orchard, maintaining a minimum distance of 10 m between each individual plant, as follows: (1) Agrícola Aguas Negras: Legacy, Elliot, Draper, Brigitta, and Duke; (2) Shine Liucura: Legacy, Elliot, and Brigitta; (3) Agroberries Asque: Brigitta, Duke, Elliot, and Legacy; (4) Agroberries Cun Cun: Brigitta, Duke, Elliot, and Legacy; (5) Agrícola Campos Álvarez: Duke and Legacy. For each labeled plant, we enclosed three clusters of flowers in the bud stage in tulle bags after removing all open flowers to ensure that only non-visited flowers remained. After the flower opening, we removed the bags and allowed a single visit to each cluster. We noted whether insects sonicated the flower during flower visits (based on our audible perception of the sound emitted by bees vibrating) and recorded information about individual plant identification, blueberry cultivar, orchard, date, and time. Immediately after an insect concluded its visit, we identified (or collected) it and marked the flower with colored wool yarn (N = 834 flowers). We also marked some non-visited flowers (N = 354 flowers) with wool yarn of a different color (control group) and re-bagged all of the flowers. We, then, killed the collected insects and stored them in individually labeled flasks. In the laboratory, we photographed and identified the insects to the lowest taxonomic level possible.

We kept the flowers bagged for approximately 70 (±3) days and then checked whether the flowers set fruit, and if they did, we immediately weighed each fruit using a precision balance (Dblue® model DBPDWS32S, Valparaíso, Chile; precision 0.001g). In the laboratory, we separated the seeds from the pulp and counted the number of viable seeds (according to Dogterom et al., 2000; Rogers et al., 2013).

2.3. Statistical analysis

We arranged the data according to visitor taxon and according to the occurrence of buzzing behavior during each visit. Data were not normally distributed according to visual inspection with the QQ plot and Shapiro–Wilk tests, so we used non-parametric tests.

We used generalized linear mixed models (GLMMs) to determine whether the fruit set (binomial distribution, link = "logit") differed among floral visitor taxa and the control (non-visited flowers) (function "glmmTMB", "glmmTMB" package, Brooks et al., 2017). We built another GLMM to determine whether fruit sets differed between visits with and without sonication, among study orchards, and blueberry cultivars. Individual plants (10 individual plants per blueberry cultivar/orchard) were included as the random factor for these models.

To analyze how fruit weight (response variable) was influenced by pollinator taxon, visits with and without sonication, study orchard identification, blueberry cultivar, and the interaction between pollinator taxon and blueberry cultivar, we used a GLMM with a gamma distribution using the glmmTMB function in the glmmTMB package (Magnusson et al., 2019). To analyze if the number of seeds per fruit (count response variable) was influenced by pollinator taxon, sonication, blueberry cultivar, and the interaction between pollinator taxon and blueberry cultivar, we used a GLMM with a zero-inflated negative binomial distribution ("nbinom1") using the glmmTMB function in the glmmTMB package (Magnusson et al., 2019). We included the orchard name (as shown in Table 1) as a random factor for these models.

We assessed the fit of all models using the diagnostic plots of the "DHARMa" package (Hartig, 2020). We tabulated estimated marginal means for each visitor taxon using the R package "emmeans" (Lenth, 2019) and tested the significance of pairwise differences by the Tukey method. We specified the type II Wald chi-square ($\chi 2$) tests via the Anova() function in the car package (Fox et al., 2018). We performed all statistical analyses with R software version 4.0.3 (R Core Team, 2017).

3. Results

During 1,056 h of sampling effort for the single-visit test, we recorded 927 visits of 18 insect species to flowers of highbush blueberry cultivars in the five studied orchards. Among the visiting species, 15 were native and three were exotic/managed. Buzzing behavior (sonication) occurred in 45.6% of all visits. Only bees were observed performing buzzing behavior, and among them, the native species were *Cadeguala occidentalis* (sonicated in 68.4% of visits) and *Colletes cyanescens* (sonicated in 50% of visits), and one managed species was *Bombus terrestris* (sonicated in 56.7% of visits). We pooled as "other native bees" (sonicated in 9.1% of visits) the native bee species that did not reach a minimum of 10 samples as follows: *Corynura chloris*, *Corynura* sp., *Centris cineraria*, *Caenohalictus* sp., and *Ruizantheda proxima*. Moreover, two native (*B. dahlbomii* and *C. nigritulus*) and two exotic (*B. ruderatus* and *A. mellifera*) bee species were not observed performing buzzing behavior. Similarly, the remaining floral visitor species (three flower flies, Syrphidae) were not observed performing buzzing behavior during visits to blueberry flowers. We pooled in "syrphids" all the flower flies because none of them reached the minimum of 10 samples (Figure 1).

3.1. Fruit set

Overall, 60.4% of all visited flowers and 33.6% of unvisited flowers (bagged flowers, control) of highbush blueberry set fruit. However, the fruit set was influenced by the floral visitor taxon (glmmTMB: $\chi^2 = 19.4$, df = 10, $p < 0.05$, $N = 1281$). Visits of both native bees (especially *B. dahlbomii*, *C. cyanescens*, *C. occidentalis*, *C. nigritulus*, and "other native bees") and exotic/managed bees (*B. ruderatus*, *B. terrestris,* and *A. mellifera*) produced proportionally more fruits than the control (Figure 1). On the other hand, visits by syrphids set fewer fruits than the control (Figure 1). However, none of these contrasts were statistically significant, except that those flowers visited by *C. occidentalis* and *B. terrestris* set more fruits than those visited by syrphids (Supplementary Table S2).

Similarly, the fruit set was also dependent on the type of blueberry cultivar (glmmTMB: $\chi^2 = 12.89$, df = 4, $p < 0.01$): 75.2% and 76.5% of all tested flowers of Elliott and Duke, respectively, featured fruit set, while 56.9%, 50%, and 43.6% of the flowers of Legacy, Draper, and Brigitta featured fruit sets, respectively. However, only the contrast between Draper and Elliott presented a significant difference (Odds ratio = 0.13, t-ratio = −2.838, p = 0.0374; Supplementary Table S3). However, the presence/absence of sonication behavior (glmmTMB: $\chi^2 = 0.44$, df = 1, $p = 0.5074$) and orchard ID (glmmTMB: $\chi^2 = 3.35$, df = 3, $p = 0.3403$) did not influence fruit set.

3.2. Fruit weight

Fruit weight for single-visited flowers of highbush blueberry depended on floral visitor taxon (glmmTMB: $\chi^2 = 99.5$, df = 7, $p < 0.001$, $N = 481$). Flowers visited by *C. occidentalis* (Mean ± SD; 1.20 ± 0.87g, $N = 213$) set the heaviest fruits, followed by those visited by *B. terrestris* (0.86 ± 0.59g, $N = 191$) and *A. mellifera* (0.76 ± 0.78g, $N = 83$; Figure 2). However, there were no significant differences among these three bee species. Additionally, flowers visited by any of these species produced heavier fruits than unvisited flowers (control, 0.40 ± 0.34g, $N = 119$; Supplementary Table S4). On the other hand, flower visits of the native bees *Corynura herbsti* and *Lasioglossum* sp. resulted in lighter fruits than those of the control; visits of "other native bees" set fruits with no difference in weight from those of the control.

Fruit weight was also influenced by the blueberry cultivar (glmmTMB: $\chi^2 = 219.389$, df = 4, $P < 0.001$). Elliott set the lightest fruits among all cultivars, followed by Brigitta with fruits approximately two times heavier than Elliott. The fruits of Brigitta did not differ from those of Legacy (Supplementary Table S5). Duke and Draper set the heaviest fruits with no difference between them (Supplementary Table S5). The fruits of Duke and Draper could be on average 4.3 times heavier than those from Elliott and almost double the weight of the fruits of the Legacy cultivar (Supplementary Table S5).

Fruit weight was also influenced by the interaction between pollinator taxon and blueberry cultivar (glmmTMB: $\chi^2 = 62.3$, df = 12, $P < 0.001$; Figure 2). The flowers of Brigitta visited by "other native bees" (composed mostly of small halictid bees) set lighter fruits than those visited by *C. occidentalis* and the unvisited flowers (control, Table 2; Figure 2). Comparisons among the other pollinator taxa and control were not significant for the Brigitta cultivar (Supplementary Table S6).

The flowers of the Duke cultivar visited by any bee species set heavier fruits than the control of unvisited flowers (Figure 2, Table 2). The floral visits of *C. occidentalis* produced the heaviest fruits of Duke with a significant difference from other bee species, except *B. terrestris*. The other comparisons were not statistically significant (see Figure 2; Supplementary Table S6). In the case of the Elliott cultivar, only pollination by *C. occidentalis* and "other native bees" resulted in heavier fruits than the control (Table 2); flowers visited by *A. mellifera* set lighter fruits than those of *C. occidentalis* (Figure 2). The other comparisons for Elliott were not significant (Table 2). Finally, the pollinator taxon did not influence fruit weight, and no contrasts were significant for the Legacy cultivar (Figure 2; Supplementary Table S6).

Fruit weight did not differ significantly between flowers visited with and without buzzing behavior (glmmTMB: $\chi^2 = 0.90$, df = 1, $P = 0.3419$).

3.3. Seed set

Seed set varied according to pollinator taxon (glmmTMB: $\chi^2 = 122.03$, df = 4, $P < 0.001$, $N = 595$), highbush blueberry cultivar type (glmmTMB: $\chi^2 = 127.10$, df = 3, $P<0.001$, $N = 595$), and the interaction between these two variables (glmmTMB: $\chi^2 = 30.2$, df = 12, $P<0.005$, $N = 595$). Flowers pollinated by *B. terrestris* (Mean+SD: 21.7 ± 12.6) and *C. occidentalis* (10.3 ± 11.1 seeds/fruit) set fruits with more seeds than unvisited flowers (control, Figure 3), without a difference between them (Supplementary Table S7). Flowers pollinated by these two bees set fruits with more seeds than did flowers

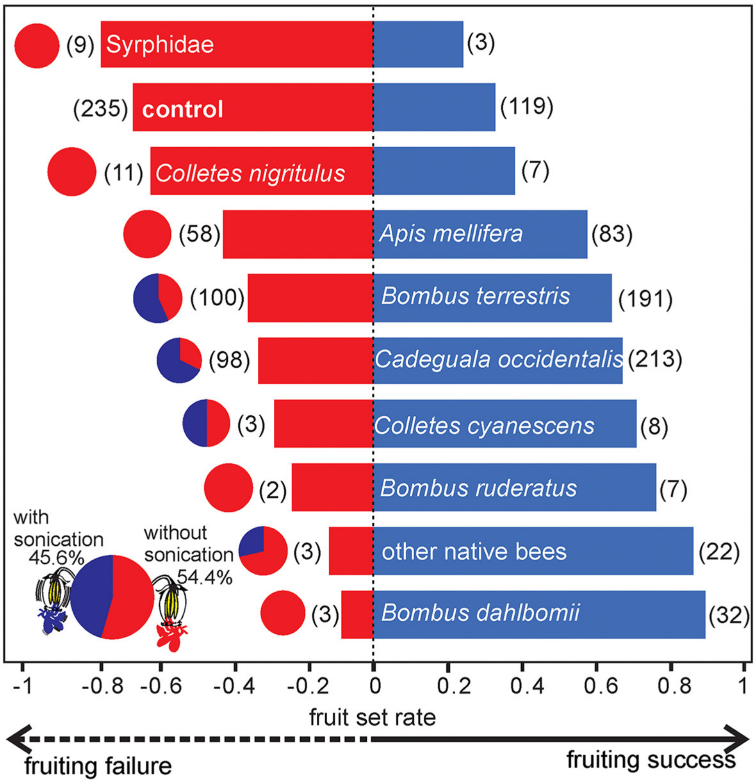

FIGURE 1
Proportion of fruit set failure (flowers that did not convert into fruits, red colored) and fruit set success (flowers that converted into fruits, blue colored) of single-visited flowers of highbush blueberry cultivars in five orchards in the southern region of Chile. The pie charts represent the proportion of visits with floral sonication (blue) and without (red) in relation to all visits observed of a visitor species. The number between brackets indicates the number of individuals observed (N) per species. Other native bees include bees that did not reach ten samples: *Corynura chloris*, *Corynura* sp., *Centris cineraria*, *Caenohalictus* sp., *Ruizantheda proxima*.

pollinated by *A. mellifera* (3.4 ± 5.3 seeds per fruit) and "other native bees" (3.4 ± 5.3). In addition, flowers visited by *A. mellifera* and "other native bees" set fruits with a similar number of seeds to those of the control (Supplementary Table S7); here, "other native bees" includes *B. dahlbomii* because it did not reach the minimum number of samples ($N < 10$).

Legacy was the cultivar that produced the greatest seed set per fruit among all the tested cultivars, while Brigitta produced the least (Supplementary Table S8). The remaining cultivars (Duke and Elliott) did not differ significantly for the seed set (Supplementary Table S8).

The interaction between the blueberry cultivar and pollinator taxon was also significant (glmmTMB: $\chi^2 = 30.2$, df = 12, $P < 0.005$, $N = 595$; Figure 3). Flowers visited by *B. terrestris* set more seeds than the control for all four cultivars (Table 3). However, flowers visited by *C. occidentalis* set more seeds than the control in three of the four cultivars (except Legacy, Table 3, Supplementary Table S9). Despite visits of *B. terrestris* resulting in a greater average seed set, there was no difference with *C. occidentalis* among the blueberry cultivars (Supplementary Table S9). On the other hand, "other native bees" only showed a positive effect compared to the control for the Elliott cultivar and presented a negative effect or no difference when compared to the control for the other cultivars.

3.4. Correlation between seed set and fruit weight

All cultivars presented a strong positive correlation between seed set and fruit weight (Figure 4). However, the influence of seeds per fruit was stronger for Brigitta ($R = 0.53$, $p < 0.001$) and Duke ($R = 0.79$, $p < 0.001$), while for Elliot ($R = 0.64$, $p < 0.001$) and Legacy ($R = 0.57$, $p < 0.001$), it was weaker, especially for Legacy, which produced the largest seed set but without a proportional increase in fruit weight (Figure 4).

4. Discussion

Despite the occurrence of floral sonication being a predictor of higher pollen deposition on the stigmatic surface (see Cortés-Rivas et al., 2023), we found that the occurrence of this behavior alone was not a predictor of higher fruit set and fruit weight of highbush blueberry cultivars. Instead, fruit sets and fruit quality improvement are more dependent on the taxonomic identity of the visiting species. Among them, some native bees can greatly improve the fruit set and fruit quality (greater weight) of highbush blueberry cultivars. Therefore, the taxonomic identity of floral visitors, ideally at the species level, is still needed to distinguish the most efficient promoters of blueberry fruit yield.

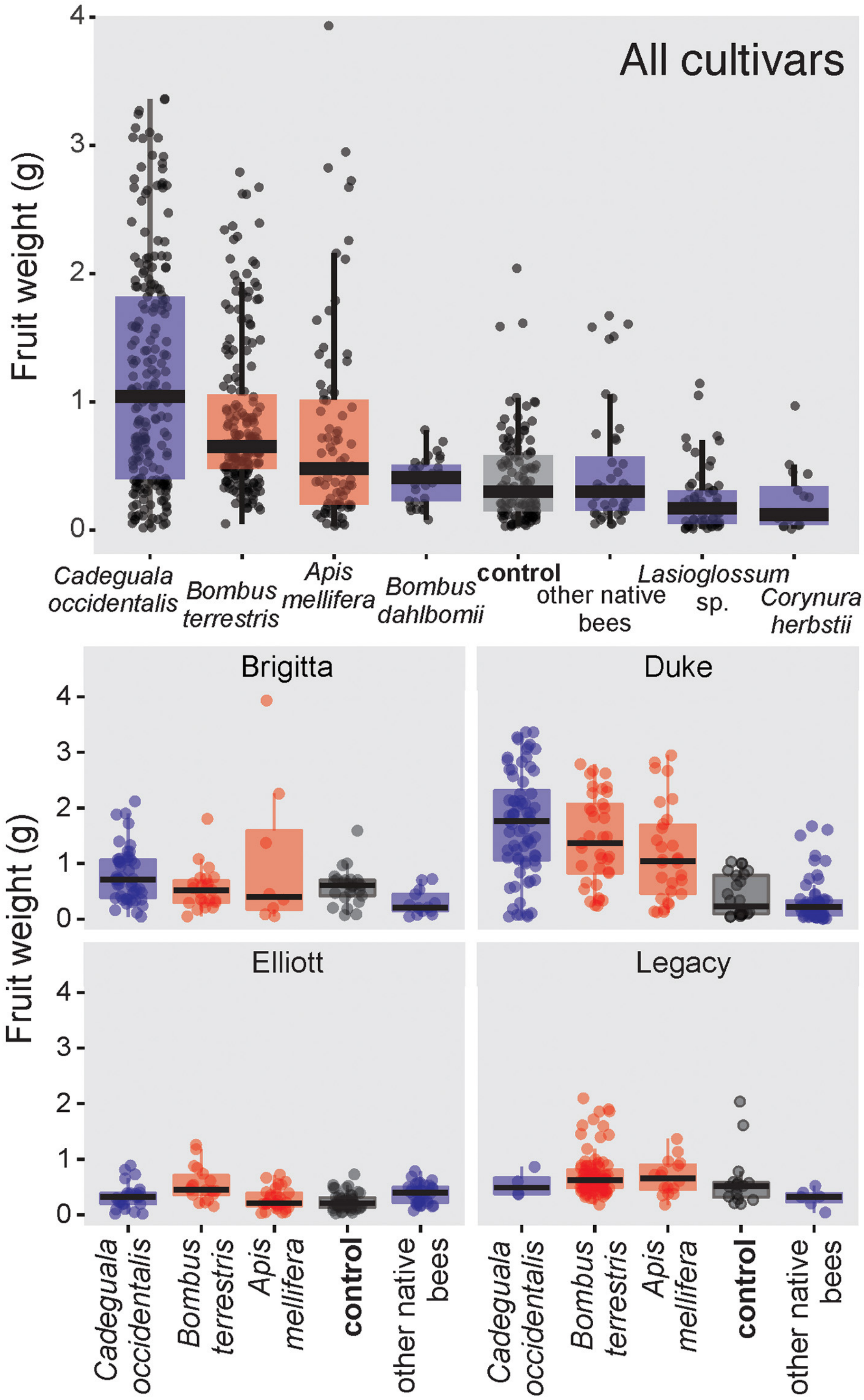

FIGURE 2
Boxplots comparing the effect of the pollinator taxon on the highbush blueberry fruit weight (grams) based on single-visit tests in five orchards in the southern region of Chile. The top graphic considers the effect of pollinator taxon on fruit weight of all blueberry cultivars together, and the four bottom graphics show the effect per highbush blueberry cultivar. The boxes are colored according to the geographic origin of the visiting insects: native (blue), exotic (red), and control (black). Other native bees include bees that did not reach ten samples: *Corynura chloris, Corynura sp., Centris cineraria, Caenohalictus sp., Ruizantheda proxima, Colletes cyanescens, Colletes nigritulus*.

TABLE 2 Pairwise contrasting of the interaction between visitor taxon and highbush blueberry cultivar for fruit weight in five orchards of the southern region of Chile (gamma distribution, glmmTMB).

Contrast	Ratio (±SE)	*t*-ratio	*p*-value
Blueberry cultivar = Brigitta			
Control vs. other native bees	2.20 (±0.56)	3.117	0.0164
C. occidentalis vs. other native bees	2.86 (±0.75)	3.976	0.0007
Blueberry cultivar = Duke			
Control vs. *A. mellifera*	0.28 (±0.05)	−6.413	<0.0001
Control vs. *B. terrestris*	0.23 (±0.05)	−6.429	<0.0001
Control vs. *C. occidentalis*	0.16 (±0.04)	−7.784	<0.0001
Control vs. other native bees	0.47 (±0.12)	−2.933	0.0287
A. mellifera vs. *C. occidentalis*	0.59 (±0.11)	−2.830	0.0385
C. occidentalis vs. other native bees	2.82 (±0.72)	4.077	0.0005
Blueberry cultivar = Elliott			
Control vs. *C. occidentalis*	0.40 (±0.09)	−4.135	0.0004
Control vs. other native bees	0.55 (±0.10)	−3.186	0.0131
A. mellifera vs. *C. occidentalis*	0.50 (±0.12)	−2.788	0.0433
Blueberry cultivar = Legacy			
None of the contrasts was significant			

Information about the geographical location, farming type, cultivated area, blueberry cultivars, and bee species managed for crop pollination.

4.1. Chilean native bees can enhance the fruit set and quality of blueberry crops

As previously expected, the exclusion of biotic pollinators reduced the fruit set of highbush blueberry cultivars. However, the capacity of floral visitors to pollinate varied greatly, with only a subset of all floral visitors actually pollinating the flowers and promoting the fruit set. The flowers that were single-visited by native and managed bees, especially bumblebees (*Bombus dahlbomii*, *B. ruderatus*, and *B. terrestris*) and medium-sized solitary bees (*C. cyanensis*, *C. occidentalis*, and other native bees), set a higher number of fruits compared to the control of unvisited flowers, and are likely to increase fruit set. Among them, the giant Patagonian bumblebee (*B. dahlbomii*) achieved the highest proportion of fruit sets, with more than 90% of flowers visited setting fruits.

The high efficiency to set fruits of blueberry could be a new piece of evidence to enhance the functional role of *B. dahlbomii* as a relevant crop pollinator and highlights the urgent need to implement conservation strategies because of the delicate conservation status of this bumblebee (Morales et al., 2013; Smith-Ramírez et al., 2018; Henríquez-Piskulich et al., 2021). This bumblebee is classified as a threatened species by the IUCN Red List with declining populations (Morales et al., 2016), and its threat has been attributed to, among other factors, the introduction of *B. terrestris* hives for crop pollination, especially blueberries (Morales et al., 2013; Smith-Ramírez et al., 2018). Therefore, strategies to enhance crop pollination with this exotic species necessarily need to deal with the fragile conservation status of *B. dahlbomii*. These strategies could include integrative habitat management and more sustainable alternatives to replace the importation of *B. terrestris* colonies.

In addition to the effect on fruit set, fruit quality and seed set were strongly pollinator-dependent. Fruits from flowers visited by pollinators can be three times heavier and set up to 6.3 times more seeds than those from non-visited flowers. This is particularly relevant for the farmer since fruit quality (heavier and larger fruits) has direct consequences on market price (Gilbert et al., 2014; Retamales and Hancock, 2018; Hall et al., 2020). The ground-nesting solitary bee *C. occidentalis* achieved high performance as a pollinator of blueberry and is similar to the exotic and managed bumblebee (*B. terrestris*). Both of these species are sonicating bees. Surprisingly, we found that a single visit of *C. occidentalis* can increase fruit weight by 5.8 times over that of non-visited flowers and 1.8 times over those visited by *A. mellifera*. *Cadeguala occidentalis,* commonly found visiting other crops (Monzón et al., 2020; López-Aliste et al., 2021; Smith-Ramírez et al. in review), is widely distributed in Chile (GBIF Secretariat, 2021) and has known nesting behavior and biology (Torchio and Burwell, 1987; Montalva et al., 2011; López-Aliste et al., 2021). However, key aspects of the biology of *C. occidentalis*, especially its solitary behavior and ground-nesting, need to be taken into consideration to enhance blueberry pollination with this bee species. Grazing, for example, can destroy nest sites, remove forage plants, and alter the structure, diversity, and growth habits of the plant community (Black et al., 2011). Despite prescribed burns being beneficial for maintaining some ecosystems, they also significantly reduce ground-nesting bee populations (Black et al., 2011).

4.2. Floral sonication alone is not a predictor of high fruit yield

Despite the occurrence of buzzing behavior (or floral sonication) being related to higher conspecific pollen deposition on stigmas of highbush blueberry (Cortés-Rivas et al., 2023), we found that floral sonication did not have significant effects on fruit set and fruit weight. Instead, fruit weight was particularly dependent on pollinator taxon, even more than on the presence/absence of buzzing behavior. Buzzing behavior is widespread and occurs among many bee taxa, including bees that differ greatly in morphology and foraging behavior (Cardinal et al., 2018). For example, some small bees capable of performing buzzing behavior (*Lasioglossum* sp. and *C. chloris*) were less efficient pollinators of the studied highbush blueberry orchards, their visits only resulting in low or no fruit set. This must be, among other factors, because their body is not large enough to touch the stigmatic region while searching for nectar and pollen on the flower (Solís-Montero and Vallejo-Marín, 2017; Földesi et al., 2021). Therefore, bee body size relative to blueberry flower size and foraging behavior must be among the key co-factors affecting pollination efficiency

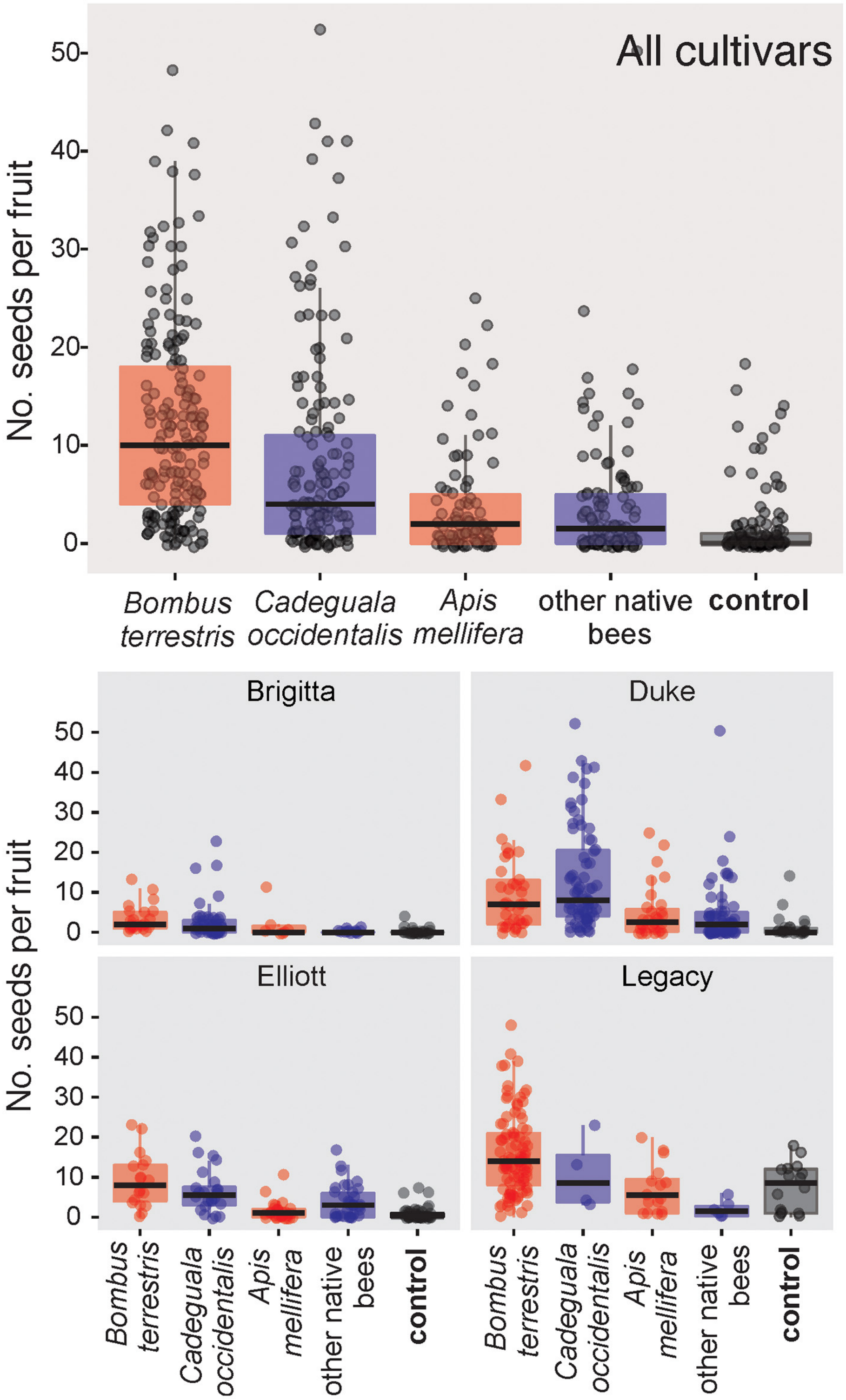

FIGURE 3
Boxplots comparing the effect of the pollinator taxon on the seed set of highbush blueberry cultivars based on single-visit tests in five orchards in the southern region of Chile. The top graphic considers the effect in all blueberry cultivars together, and the four bottom graphics show the effect per highbush blueberry cultivar. Boxes are colored according to the geographic origin of the visiting insects: native (blue), exotic (red), and control (black). "Other native bees" includes bees that did not reach ten samples: *Bombus dahlbomii, Corynura chloris, Corynura sp., Centris cineraria, Caenohalictus sp., Ruizantheda proxima, Colletes cyanescens, Colletes nigritulus, Lasioglossum sp., Corynura herbsti.*

(Solís-Montero and Vallejo-Marín, 2017; Morais et al., 2020; Földesi et al., 2021; Mesquita-Neto et al., 2021). The bees that were notably equal to or larger in size than the anther–stigma distance achieved greater performance at setting fruit with the highest weight (e.g., *B. terrestris*, *B. dahlbomii*, *C. occidentalis*, and *Colletes* spp.). However, we did not measure the body size of individual bees nor the anther–stigma distance of blueberry flowers, which could be a better predictor of fruit yield than the buzzing behavior alone and could be addressed by further studies. Therefore, our results cannot support our hypothesis that the buzzing behavior alone is a predictor of higher fruit set and weight. Instead, species identity remains a better predictor of the quantity and quality of blueberry fruits.

Like bees, some syrphids (flower flies) can generate vibrations of sufficient amplitude to extract pollen from buzz-pollinated flowers (Vallejo-Marín and Vallejo, 2021). However, we have not observed flies performing floral sonication in blueberry flowers. In fact, only one case of floral sonication by flower flies has been reported, involving a Mexican cactus fly (*Volucella mexicana*) in the flowers of a *Solanum* plant (Buchmann et al., 1978). The absence of this behavior is explained by the fact that the amount of pollen required by a flower fly is much less than that required by a bee (Larson et al., 2001). Syrphids usually do not feed their offspring with pollen, and their larvae consume other resources (Diaz et al., 2020). Pollen consumption is, then, only related to the diet of adult flies (Larson et al., 2001). In addition, there is very little information on the role of flower flies and other flies in the pollination and fruit production of blueberries in Chile or elsewhere. (Cook et al., 2020; Cortés-Rivas et al., 2023). Mann (2014) suggested that blowflies (Calliphoridae) may be effective pollinators due to their ability to sonicate, but no direct evidence of pollination efficiency was presented. To our knowledge, only the study by Cook et al. (2020) provides evidence that the presence of blowflies in greenhouses improves the pollination of blueberries. However, Cortés-Rivas et al. (2023) showed that flower flies were poor pollinators of blueberry plants, depositing few pollen grains on the stigmatic surface per visit. Our results showed that their visits resulted in a similar proportion of fruit compared with unvisited flowers. Although flies are capable of producing floral vibrations, our study provides evidence against the notion that flower flies are effective pollinators of highbush blueberry cultivars but are rather pollen or nectar thieves.

TABLE 3 Pairwise contrasting of the effect of the interaction between visitor taxon and highbush blueberry cultivar on seed set in five orchards of the southern region of Chile (nbinom1 distribution, glmmTMB).

Contrast	Ratio	*t*-ratio	*p*-value
Blueberry cultivar = Brigitta			
Control vs. *B. terrestris*	0.15 (±0.08)	−3.446	0.0055
Control vs. *C. occidentalis*	0.18 (±0.09)	−3.286	0.0095
Blueberry cultivar = Duke			
Control vs. *A. mellifera*	0.28 (±0.11)	−3.149	0.0148
Control vs. *B. terrestris*	0.16 (±0.06)	−4.797	<0.0001
Control vs. *C. occidentalis*	0.12 (±0.04)	−5.743	<0.0001
A. mellifera vs. *C. occidentalis*	0.43 (±0.09)	−3.899	0.0010
B. terrestris vs. other native bees	2.45 (±0.67)	3.257	0.0104
C. occidentalis vs. other native bees	3.22 (±0.82)	4.585	0.0001
Blueberry cultivar = Elliott			
Control vs. *B. terrestris*	0.17 (±0.05)	−5.481	<0.0001
Control vs. *C. occidentalis*	0.17 (±0.05)	−5.629	<0.0001
Control vs. other native bees	0.27 (±0.09)	−4.101	0.0004
A. mellifera vs. *B. terrestris*	0.25 (±0.08)	−4.358	0.0002
A. mellifera vs. *C. occidentalis*	0.26 (±0.08)	−4.348	0.0002
A. mellifera vs. other native bees	0.41 (±0.13)	−2.810	0.0407
Blueberry cultivar = Legacy			
Control vs. *B. terrestris*	0.51 (±0.12)	−2.905	0.0311
B. terrestris vs. other native bees	4.34 (±21.16)	3.013	0.0226

4.3. Blueberry pollination, sustainable agriculture, and pollinator conservation

Buzz-pollinated plants and their flower visitors represent a tangible example of the importance of considering bee functional diversity in the pollination of cultivated species (Cooley and Vallejo-Marín, 2021). However, our results evidenced that taxonomic diversity is also a key component since the quality of the pollination provided also depends on the taxon of flower visitors. Consequently, the taxonomic recognition of species becomes indispensable to distinguishing the most efficient pollinators of blueberry (Cortés-Rivas et al., 2023). Therefore, by discriminating true fruit set promoters from nectar/pollen thieves and being aware of the value of bees to crop income, farmers could be encouraged to consider the pollination perspective in their crop management. This could result in the conservation of local wild bee species, thereby contributing to advances toward more sustainable and higher-yield agriculture (Isaacs and Kirk, 2010; Garibaldi et al., 2014; Nicholson and Ricketts, 2019).

Our results showed that some native Chilean pollinating bees (e.g., *B. dahlbomii* and *C. occidentalis*) can be much better fruit yield promoters than *A. mellifera* and equivalent to the exotic bumblebee. However, the potential to enhance blueberry fruit production alone does not justify the immediate abandonment of managed hives to pollinate blueberry cultivars. There are practical challenges associated with supplementing pollination with native pollinators (Cooley and Vallejo-Marín, 2021). One of them is increasing their abundance in crop fields (Isaacs and Kirk, 2010; Garibaldi et al., 2014; Henríquez-Piskulich et al., 2021), as they represented only 11.9% of all visits in the studied blueberry orchards (Cortés-Rivas et al., 2023). Strategies to increase native pollinators can include creating wildflower strips for pollinators and the restoration of hedgerows on farms, along with the conservation of nearby natural and semi-natural habitats (Morandin and Kremen, 2013; Potts et al., 2016; Sutter et al., 2018; Rodríguez et al., 2021). Such efforts would provide nesting habitats and floral resources for the most effective pollinators of blueberry and consequently, the overall native bee community within agricultural ecosystems since they not only visit crops but also depend on native habitats to nest, feed, and reproduce (Nicholson et al., 2017; Monzón and Ruz, 2018; Rodríguez et al., 2021). Second, attention must be taken to ensure that commercial colonies of native bees do not become a problematic resource in their native range (Mallinger et al., 2017). Despite that, we expect that our study will be one more piece of evidence to discourage the importation of *B. terrestris* hives for blueberry pollination in Chile while incentivizing the development

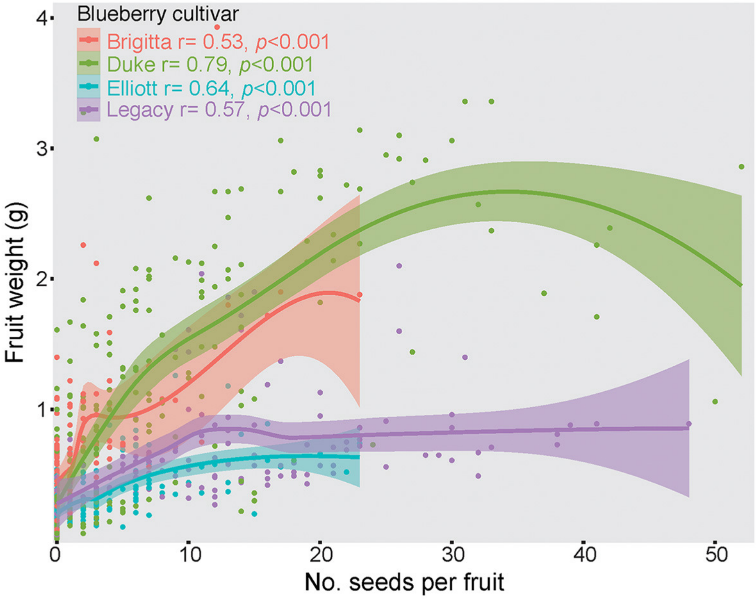

FIGURE 4
Non-linear correlation between the number of seeds per fruit and the fruit weight (grams) of four highbush blueberry cultivars in five orchards southern region of Chile.

and implementation of integrative habitat management strategies to conserve native bees and consequently enhance crop pollination.

To our knowledge, this is the first study evidencing the per-visit performance of native crop pollinators to enhance fruit quantity and quality in the Chilean environment. Here, we applied field experimental protocols and sampling methods to investigate blueberry pollination and evaluated the relative performance of managed bees and native bees in per-visit-based tests in commercial blueberry fields. Bees, however, vary in their foraging range (Greenleaf et al., 2007; Zurbuchen et al., 2010) and distribution, so examining the bees that visit blueberries over multiple regions and landscape scales can help reveal how these factors affect pollinators and their pollination services. Moreover, sampling bias must be another relevant factor, since a limited number of flowers set fruit. Considering that a plant is typically visited by multiple flower visitors and per visitor replicates are needed, replicates were missing for some flower visitor species.

In summary, our results indicate that visits of native bees can enhance fruit production of highbush blueberry crops in Chile. However, fruit set and fruit quality improvement are more dependent on the taxonomic identity of the visiting species rather than their sonication behavior alone. Some native Chilean bees, especially *C. occidentalis* and the giant Patagonian bumblebee (*B. dahlbomii*) were efficient fruit promoters of blueberry and performed better or similar to honeybees (*A. mellifera*) and the exotic bumblebee. We suppose that the conservation of these native pollinators would result in increased crop yields because their visits enhance blueberry fruit quality and are likely to improve overall crop productivity and sustainability. Therefore, instead of continuing to rent or pay for managed beehives, which represents a high cost for farmers (Isaacs and Kirk, 2010; Henríquez-Piskulich et al., 2021), the service provided by native pollinators should be considered, with priority given to the subset of the most successful pollinators.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

BC-R, JM-N, and VM contributed to the conception and design of the study. BC-R, JR, and JM-N carried out the experiment. JM-N organized the database and performed the data and statistical analysis. BC-R and JM-N wrote the first draft of the manuscript. JR and VM wrote sections of the manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1142623/full#supplementary-material

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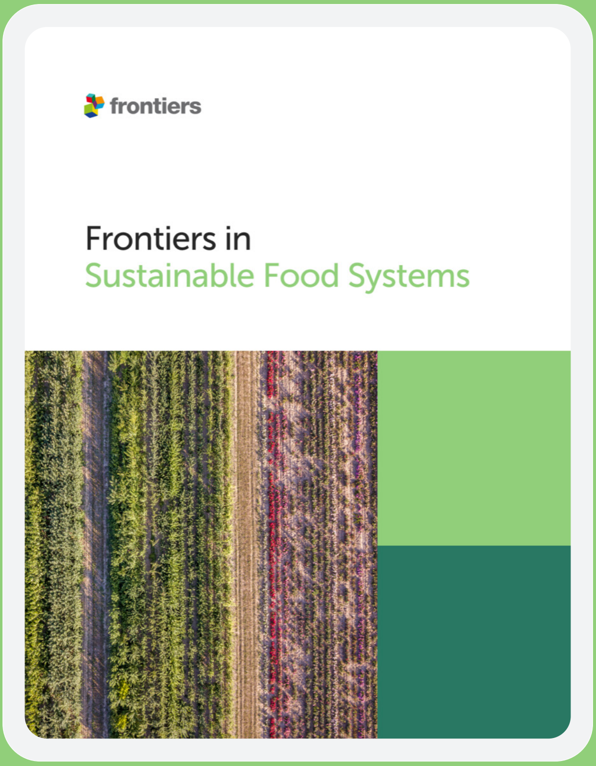

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